THE ALANINE, CYSTINE, GLYCINE AND SERINE CONTENT OF MEAT[1]

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TWO FIGURES

(Received for publication July 13, 1953)

Microbiological methods are widely used for the determination of amino acids in natural products. Previous papers from this laboratory (Schweigert et al., '45; Greenhut et al., '47, '48; Sirny et al., '50; Violante et al., '52) have reported the amino acid content of various cuts of meats for 14 of the 18 common amino acids, determined microbiologically.

The methods employed in the previous work have been extended to determination of the 4 other common amino acids — alanine, cystine, glycine and serine. Alanine was determined with *Leuconostoc citrovorum* 8081, cystine and glycine with *L. citrovorum* and *Leuconostoc mesenteroides* P-60, and serine with *Lactobacillus casei* and *Lactobacillus delbrueckii* 3.

EXPERIMENTAL

The meats employed for these determinations were the same samples as those described by Violante et al. ('52). The medium of Henderson and Snell ('48), with some modification, was used. As recommended by Chitre et al. ('51), a low sodium content was maintained. To eliminate

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by funds supplied by the National Live Stock and Meat Board, Chicago, Illinois.

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the variations due to salt concentrations reported by Camien et al. ('47) and confirmed by tests in this laboratory, the salt concentration of the standard was adjusted to be equivalent to that found in the meat hydrolysate when the pH was adjusted to 6.8 (Chitre et al., '51; Violante et al., '52). Leucovorin [2] was added to the medium for *L. citrovorum* at the rate of 0.02 μg per tube to replace the Reticulogen used by Steele et al. ('49). A pH of 6.8 was used in all assays. Tween 80 (polyoxyethylene sorbitan monooleate) was added to all assay media at a level of 0.005% by volume (Alexander and Hill, '52). In the assay for serine, the threonine content was reduced to one-fourth of that in the standard medium, as the microorganisms *L. casei* and *L. delbrueckii* do not require threonine for growth and are inhibited by high levels of this amino acid. This inhibition is counteracted by adding $CaCl_2 \cdot 2H_2O$ to the media at the rate of 0.5 mg per milliliter for *L. casei* and 0.1 mg per milliliter for *L. delbrueckii*.

The assays were conducted with a final volume of 2 ml per tube, employing the Cannon automatic dispenser and titrator. The standard solutions were prepared to contain, per milliliter, 30 μg of DL-alanine, 5 μg of L-cystine, 20 μg of glycine and 40 μg of DL-serine. In confirmation of a report by Sauberlich and Baumann ('49), *L. citrovorum* also utilizes both isomers of DL-alanine equally well under our assay conditions. Therefore, the values reported in the tables were calculated to represent only the naturally-occurring L-isomer. The assays were sterilized by autoclaving for 7 minutes at 115°C.

Inocula were from 5-ml broth cultures grown from microorganisms carried on nutrient agar and transferred 18 to 20 hours prior to inoculation. After centrifuging, the cells were resuspended in 15 ml of 0.9% KCl and each tube received one drop of the suspension aseptically from a syringe. All tubes were incubated at 37°C. for 68 to 72 hours and then titrated with 0.1 N NaOH.

[2] Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York.

TABLE 1

Recovery values and release of amino acids from beef round with varying time of hydrolysis[1]

HYDROLYSIS TIME[2]	ALANINE	CYSTINE		GLYCINE		SERINE	
	L. citrov.	*L. citrov.*	*L. mes.*	*L. citrov.*	*L. mes.*	*L. d. 3*	*L. casei*
hours							
1	5.4	0.87	0.75	4.2	4.2	4.0	4.5
2	5.4	0.89	0.89	4.3	4.2	4.3	4.4
3	5.6	0.87	0.87	4.3	4.1	4.3	4.5
5	5.5	0.74	0.78	4.1	4.4	4.3	4.2
10	6.2	0.56	0.75	4.5	4.6	4.2	4.1
15	5.8	0.48	0.66	4.4	4.4	4.1	3.9
25	5.6	0.35	0.50	4.3	4.2	4.1	2.9
Average % recovery, 3 hours hydrolysate	98	98	100	100	99	98	99
No. of assays for recovery	6	5	5	4	4	4	4

[1] Expressed in grams per 100 gm of protein calculated to 16% N.
[2] In 3 N hydrochloric acid at 121°C.

TABLE 2

Analysis of meats[1]

CUTS OF MEAT	% CRUDE PROTEIN[2] (N × 6.25)	ALANINE	CYSTINE		GLYCINE		SERINE	
		L. citrov.	*L. citrov.*	*L. mes.*	*L. citrov.*	*L. mes.*	*L. d. 3*	*L. casei*
Veal								
Round	82.9	6.3	1.0	1.0	4.6	4.7	4.7	4.9
Shoulder	85.3	6.1	1.0	1.0	4.6	4.6	4.5	4.6
Lamb								
Leg	82.3	6.2	1.0	1.0	4.4	4.6	4.9	5.0
Shoulder	82.7	6.1	1.0	1.0	4.2	4.5	4.7	4.7
Pork								
Ham	85.1	6.1	0.9	0.9	4.2	4.0	5.0	5.1
Shoulder	84.8	6.4	1.1	1.1	4.2	4.2	5.3	5.3
Beef								
Round	86.9	6.2	0.9	0.9	4.5	4.6	4.3	4.5
Shoulder	84.7	6.4	0.9	1.0	4.6	4.7	4.5	4.8
Brain	70.3	6.3	1.3	1.3	4.0	4.2	6.4	6.3
Brain	70.3	6.6	1.3	1.3	4.2	4.3	6.4	6.4
Heart	78.7	6.7	1.0	1.0	4.8	4.7	4.8	4.7
Heart	77.4	6.8	0.9	1.0	5.2	5.2	4.6	4.5
Kidney	79.7	6.2	1.3	1.3	6.2	6.0	5.4	5.4
Kidney	76.0	6.3	1.4	1.5	6.1	5.8	5.8	5.8
Liver	67.7	6.3	1.0	1.0	5.9	5.6	5.5	5.5
Liver	73.9	6.5	1.1	1.0	6.0	6.0	5.4	5.4
Tongue	83.2	6.6	1.2	1.2	6.5	6.5	5.1	5.0
Tongue	82.4	6.8	1.3	1.3	6.7	6.5	5.2	5.2

[1] Expressed in grams per 100 gm of protein calculated to 16% N.

[2] Lyophilized fat-extracted meat.

Different hydrolysis conditions for the determination of the amino acids in the lyophilized, ether-extracted meat were tested (table 1). For each 0.5 gm of sample, 20 ml of 3 N HCl were used. Preliminary studies had shown that increases or decreases in the concentration of acid did not result in higher values for these 4 amino acids. Recoveries from crystalline amino acids added to the samples prior to hydrolysis averaged from 98 to 100%. For the assay of alanine

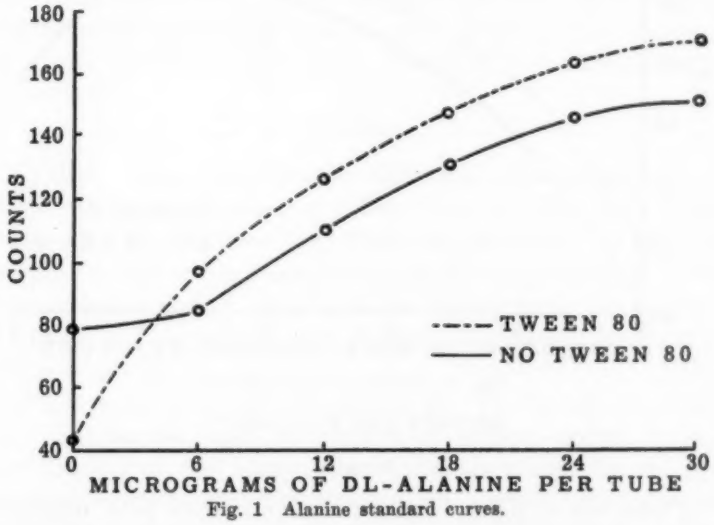

Fig. 1 Alanine standard curves.

and glycine, a hydrolysis time of 10 hours was found to give maximum liberation. A hydrolysis time of two hours was selected for the cystine assay, as beyond three hours rapid destruction of cystine was encountered under the conditions used. Most optimum results were obtained with both microorganisms for the serine assay with a hydrolysis time of three hours.

The hydrolysates were assayed at least three times by each of the two microorganisms for cystine, glycine and serine, and by one microorganism for alanine (table 2). Six

duplicate levels of each sample were read from the average of duplicate standard curves run in each rack of 60 tubes. All values are expressed in grams of amino acids per 100 gm of crude protein calculated to 16% nitrogen.

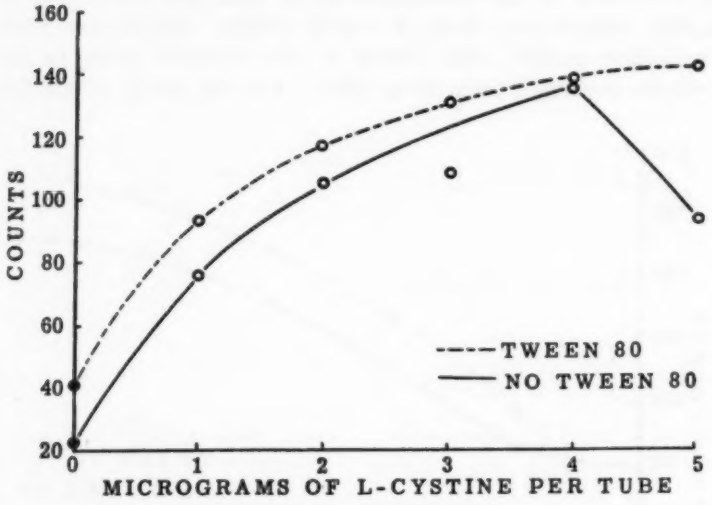

Fig. 2 Cystine standard curves.

RESULTS AND DISCUSSION

Alanine

A very satisfactory assay for alanine has been obtained by employing Tween 80 in the medium to remove a lag from the standard curve and lower blank titration values (fig. 1). It is known that lipide material such as sodium oleate lowers surface tension, and other investigators have shown that oleic acid is essential for other microorganisms (Williams et al., '47). Possibly the above effects, along with a better dispersion of the cells throughout the growth medium during the incubation period, account for this significant improvement in alanine assay conditions.

The alanine values obtained for the various cuts of meat by the use of *L. citrovorum* (table 2) ranged from 6.1% for

veal and lamb shoulder and pork ham to 6.8% for beef heart and tongue, indicating that some of the beef organs contain slightly more alanine than the muscle meats.

Sauberlich and Baumann ('49), using *L. citrovorum,* reported 4.7 and 7.4% alanine in rat liver and rat muscle, respectively. Schweigert et al. ('51), using *L. citrovorum* and *L. leichmannii,* found the alanine content of the crude protein of pork and lamb cuts to be 6.3% but reported considerable variation in graded levels of alanine, with no procedure being completely satisfactory. However, this value is in close agreement with those found by our standardized procedure.

Cystine

Cystine values determined with two microorganisms, *L. citrovorum* and *L. mesenteroides,* were in good agreement (table 2). Preliminary investigations employing *L. mesenteroides* as the test microorganism produced a standard curve with a depression of growth at the higher levels of cystine. Addition of Tween 80 to the medium eliminated the growth depression (fig. 2).

It has been reported (Riesen et al., '47; Lankford et al., '47) that considerable destruction of cystine occurs during sterilization of the assay tubes by autoclaving, due to the presence of carbohydrate in the medium. Consequently, assays were run in which the medium was added aseptically after separate autoclaving. Cystine values checked with those obtained by the conventional method, so, due to technical preference, the conventional method of sterilization was adopted.

In the case of meat samples where the carbohydrate content is very low, a nearly equivalent amount of carbohydrate is present in all tubes. With minimum autoclaving of 7 minutes at 115°C., and equal destruction in both standards and samples, this factor fails to be of major importance, as is illustrated by good recoveries of crystalline amino acids added prior to hydrolysis (table 1). Sarkar et al. ('50) found that

chemical and microbiological methods gave highly comparable assay values for the cystine content of purified proteins.

The average cystine content of muscle meats was similar for beef, veal, lamb and pork as well as for beef heart and liver, with a range from 0.9 to 1.1%. However, values for beef brain, kidney and tongue were slightly higher, ranging from 1.2 to 1.5%.

Beuk et al. ('48), using *L. mesenteroides,* reported a value of 2.7 mg of cystine per gram of fresh pork, which would be 1.2% of this amino acid expressed on the basis of 16% N.

Schweigert et al. ('51), using *L. mesenteroides,* found 1.3% cystine in pork and lamb shoulder. The same workers reported lamb leg to contain 1.2% cystine.

Glycine

The microorganisms *L. citrovorum* and *L. mesenteroides* were also very satisfactory for the glycine assay. Values for the muscle meats ranged from 4.0 to 4.7%, while beef kidney, liver and tongue showed higher values, ranging from 5.6 to 6.7%.

Schweigert et al. ('51) reported pork shoulder, lamb shoulder and lamb leg to contain 5.97, 6.59 and 7.12% glycine, respectively.

Serine

The serine contents obtained with two microorganisms, *L. casei* and *L. delbrueckii,* were in good agreement. Addition of Tween 80 to the medium for *L. casei* removed a drift from the assay values, and its addition to the medium for *L. delbrueckii* increased the recovery of crystalline amino acid from 94 to 98%.

The serine values of the muscle meats, as well as of beef heart and tongue, ranged from 4.5 to 5.3%. Beef brain, kidney and liver showed higher values, ranging from 5.4 to 6.4%.

Schweigert et al. ('51), using *L. mesenteroides,* reported lamb leg, lamb shoulder and pork shoulder to contain 3.67, 3.89 and 3.73% serine, respectively.

SUMMARY

The alanine, cystine, glycine and serine content of 18 samples of beef, lamb, pork and veal muscle cuts and beef organs have been determined by microbiological assay procedures specially modified for this purpose. Where possible, values have been compared with reports in the literature. Cystine, glycine and serine, with average values of 1.1, 5.0 and 5.2%, respectively, were each determined with two microorganisms, which gave results in good agreement with each other in repeated analyses. Alanine, determined with one microorganism, gave an average value of 6.4%. Depending on the amino acid concerned, certain of the beef organs gave higher values than the muscle meats.

Tween 80 (polyoxyethylene sorbitan monooleate) was added to all assay media at a level of 0.005% by volume. Addition of this detergent eliminated a lag from the alanine standard curve and lowered the blank titration values, removed the depression of growth for *L. mesenteroides* at the higher levels of cystine, eliminated drifts with *L. casei* in serine assays and increased the recovery of serine with *L. delbrueckii* from 94 to 98%.

In addition to the above, Tween 80 produced a general stabilizing effect in all assays, resulting in less variation and greater reproducibility of assay values.

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THE INFLUENCE OF DIETARY FAT AND CARBOHYDRATE ON GROWTH AND LONGEVITY IN RATS[1]

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ONE FIGURE

(Received for publication June 5, 1953)

Swift and Black ('49) and Deuel et al. ('47) have shown that high fat diets are superior to low fat diets for growth, efficiency of food utilization and physical capacity. There is little information, however, regarding such a comparison on a life span basis. The possibility that high fat diets may have a detrimental effect on length of life and promote the development of degenerative disease in the aged, although often expressed (Mayer, '49; Callison et al., '51), has little experimental evidence for its support.

Early work by Slonaker ('12) and Slonaker and Card ('23) indicated that restricted intake of a vegetarian type of diet resulted in a decrease in length of life for the rat. The inclusion of meat protein, however, increased the life span greatly, suggesting the possibility of a deficiency in their early diets. Later studies by Slonaker ('31, '38) on the influence of dietary protein level on growth and longevity included diets ranging in fat content from 12.2 to 19.7%. The greatest life spans were recorded on the diets lowest in protein, which were also lowest in fat and highest in carbohydrate content. Kao, Conner and Sherman ('41), however,

[1] Authorized for publication on May 7, 1953, as paper 1799 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

found no change in length of life with different protein intakes. McCay and associates ('35, '43) found that the restricted feeding of complete diets containing about 15% of fat increased the longevity of male and female rats. Supplementation with carbohydrate in the form of sucrose or milk powder tended to lengthen the life span. Carlson and Hoelzel ('47) reported that rats fed an omnivorous diet containing 28% fat and 35% protein lived somewhat longer, on the average, than those receiving a vegetarian diet containing about 8% fat and 30% protein but supplying more protein and more roughage on an isocaloric basis. It is interesting to note, however, that only one rat exceeded 1,000 days of age on the omnivorous diet, while 4 of the vegetarian group exceeded this age at death. Life span was more uniformly prolonged by intermittent fasting in the case of the rats receiving the omnivorous diet. Chang, Wu and Chen ('41), however, devised a vegetarian diet that was equal to or better than an omnivorous diet in terms of the longevity of rats.

EXPERIMENTAL

As was described in detail in a preceding paper (French et al., '52), the three diets shown in table 1 were prepared at monthly intervals, sampled for analysis and stored in a refrigerator until fed. The two experimental diets, one high in carbohydrate and one high in fat, were prepared by mixing 80% of the stock diet and 20% of sucrose or 20% of corn oil, respectively, and adding supplements of liver powder, yeast and vitamins to insure optimum levels of known and unknown factors.

The experiment extended over a period of 4 years. Each monthly mixing included all three diets prepared from one lot of commercial feed. Successive monthly mixings used new lots of feed and vitamin supplements but were fed to similar numbers of litter-mate rats distributed evenly among the three diet groups, so that minor fluctuations in the composition of the diet ingredients as time passed would not unduly influence any one group.

Food was supplied ad libitum, with weekly recording of food consumption throughout the life span of each animal, thus making possible the comparison of life span with caloric intake or efficiency of food utilization and a study of seasonal variation in food consumption. The rats were weighed at weekly intervals until they were 6 months of age and monthly

TABLE 1

Composition of diets

	STOCK	CARBOHYDRATE	FAT
	gm	*gm*	*gm*
Constituents			
Rat ration [1]	100	80	80
Sucrose	...	20	..
Corn oil [2]	...	..	20
Supplements (per 100 gm diet)			
Liver, 1–20,[3] gm	...	1.0	1.0
Yeast, brewers', gm	...	0.5	0.5
Yeast, irradiated, gm	...	0.5	0.5
Choline chloride, mg	...	160	160
Alpha-tocopherol acetate, mg	...	5	5
Thiamine hydrochloride, mg	...	0.2	0.2
Analysis			
Energy, Cal./gm	3.910	3.935	5.011
Nitrogen, %	3.77	3.09	3.11
Ether extract, %	4.35	3.38	22.72
Moisture, %	10.33	8.23	7.87

[1] Rockland Farms Rat Diet, complete.
[2] Mazola.
[3] Wilson's 1–20 liver powder.

thereafter. The environmental temperature was maintained throughout the experiment at $27 \pm 2°C.$, with few exceptions.

Consideration was given to the experiences of Carlson and Hoelzel ('47) and McCay ('49) in adopting the following procedure to insure maximum genetic uniformity and sufficient numbers of animals for satisfactory comparisons to be made. Approximately 75 male and 65 female rats for each of the

experimental diets and 40 of each sex for the stock diet group were obtained by repeated breeding of two closely related males with 8 closely related females (Wistar albino strain) and by using the second generation from these matings to complete the quota. All litters were reduced to 8 young at birth and were weaned and started on the experiment at 22 days of age.

Fifteen male and 15 female rats from each of the experimental diet groups and 7 males and 6 females from the stock diet group were sacrificed at 6 to 8 months of age for liver fat determination and histological study. It was not felt desirable to make pathologic studies at the time of death in view of the comprehensive work of Saxton ('45), but the animals were subjected to post-mortem examination when gross evidence of diseases and abnormalities was recorded. Symptoms of diseases and the time of appearance of tumors and other abnormalities were noted when observed.

The methods used for analysis of liver tissue or of the composited diet samples for energy, nitrogen, crude fat and moisture were, respectively, the bomb calorimeter, Kjeldahl procedure, Soxhlet ether extraction, and vacuum drying at room temperature over concentrated sulfuric acid.

RESULTS AND DISCUSSION

Growth and food consumption

The growth and food consumption data presented in figure 1 support previous findings from this laboratory (Forbes et al., '46; Swift and Black, '49) regarding the increased efficiency of food utilization conferred by dietary fat. The previous studies, conducted by paired feeding techniques with isocaloric food consumption, demonstrated an increased efficiency of food utilization for high fat diets by improved growth of the young rat or increased activity of the mature animal. The present data were obtained by measuring ad libitum food intakes over a much longer period, from the third to the 46th week of age. Average food consumption data be-

yond this age lose significance due to the variable occurrence of diseases and deaths. It is apparent that rats of either sex receiving the high fat diet (22.7% fat) grew fully as rapidly as those on the high carbohydrate diet (3.4% fat) despite their lower caloric intakes. This difference in food consumption amounted to 4.8 and 5.7% less total calories consumed on the high fat diet for males and females, respectively. Both experimental diets proved better than the stock diet in this regard, probably reflecting a better balance of nutrients in

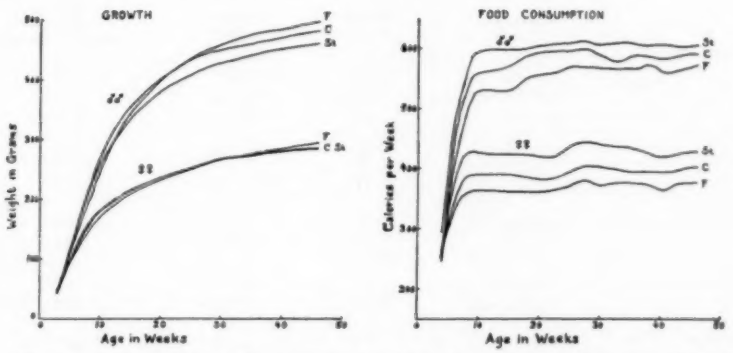

Fig. 1 Comparison of the average growth and average food consumption of 58 male and 51 female rats on the fat diet (F), 57 males and 51 females on the carbohydrate diet (C) and 32 males and 29 females on the stock diet (St).

the experimental diets and higher fiber content and lower metabolizable energy in the stock diet.

Food consumption on all diets increased rapidly to the 8th week of age for the females and to the 9th week for males and then remained relatively constant, although at a lower caloric level on the fat diet. Growth, however, continued at a rapid, although gradually decreasing, rate in both sexes until they reached 28 weeks of age or more. No great difference in growth rate due to the experimental diets was apparent until the males were more than 28 weeks of age, when the average weight of those on the fat diet began to exceed the weight of those on the carbohydrate diet by a significant

amount. By the 20th week the growth rates of males on both experimental diets were superior to that of the stock diet group, indicating that the composition of the experimental diets was nutritionally satisfactory.

In contrast to the experience of Campbell ('45), the authors noted extremely constant intakes of the diets by littermate adult animals throughout the different seasons of the

TABLE 2

Seasonal variation in food consumption of male rats

DIET	SEASON	NO. OF RATS [1]	AGE OF RATS	AVERAGE WEEKLY FOOD CONSUMED
			weeks	*gm*
Fat	April–June	17	16–20	111.9
	Oct.–Dec.	17	42–46	114.9
Fat	Oct.–Dec.	14	16–20	109.6
	April–June	14	42–46	112.8
Carbohydrate	April–June	18	16–20	145.7
	Oct.–Dec.	18	42–46	144.2
Carbohydrate	Oct.–Dec.	13	16–20	147.9
	April–June	13	42–46	155.1
Stock	April–June	10	16–20	151.7
	Oct.–Dec.	10	42–46	161.3
Stock	Oct.–Dec.	6	16–20	148.0
	April–June	6	42–46	148.8

[1] The same rats were compared with themselves in each spring and fall seasonal comparison.

year. Since the animal rooms had been maintained at a constant temperature and under similar light conditions in the winter and summer, it seemed worthwhile to examine the food consumption data. According to the hypothesis of seasonal variation in mammalian nutrition as discussed by Sargent and Sargent ('50), the cycle calls for peak consumption in the anaphase from April through June and a minimum in the cataphase from October through December. All male rats that reached the ages of 16 to 20 weeks and 42 to 46 weeks of age during either of the seasonal periods were selected, and

their average weekly food consumption in one seasonal period was compared with their corresponding food consumption during the other period. These age groups were chosen from the level sections of the food consumption curves in order to avoid periods of increasing or decreasing food consumption due to youth or old age. These data, summarized in table 2, show that no seasonal variation in food consumption occurred on any one of the three diets.

TABLE 3

Longevity

DIET	SEX	NO. OF RATS	AVERAGE AGE AT DEATH [1]	RANGE IN LONGEVITY	RANGE IN LONGEVITY OF FEMALES THAT REPRODUCED
			days	*days*	*days*
Fat	Male	58	676 ± 19 [2]	347–1,005	
	Female	51	752 ± 25	380–1,096	447–1,096
Carbo-	Male	57	765 ± 22	219–1,015	
hydrate	Female	51	793 ± 24	463–1,118	517–1,089
Stock	Male	32	747 ± 27	354– 996	
	Female	29	794 ± 31	437–1,166	610–1,004

[1] Mean and standard error of the mean.

[2] Significantly different at 1% level from either the carbohydrate or stock diet males.

Longevity, liver fat and frequency of diseases

The average ages at death and ranges in longevity presented in table 3 summarize the essential longevity data. The outstanding finding was a decreased length of life, highly significant in the male and less pronounced in the female, which resulted from ingestion of the high fat diet. Whether this decrease in longevity resulted specifically from fat as such or was mediated through the increased efficiency of food utilization and improved growth rate conferred by high fat diets, is difficult to resolve. The determination of liver fat content, the histopathological data and the frequency of diseases and abnormalities observed in the high fat and the high carbo-

TABLE 4

Liver fat content of rats 6 to 8 months of age [1]

DIET	SEX	NO. OF RATS	WEIGHT OF RAT, AVERAGE	WEIGHT OF DRY LIVER, AVERAGE	FAT CONTENT OF DRY LIVER
			gm	*gm*	%
Fat	Male	15	476	4.29	11.97 ± 0.58 [3]
	Female	15	273	2.68	7.24 ± 0.51
Carbohydrate	Male	15	470	4.26	6.71 ± 0.33
	Female	15	298	2.90	5.67 ± 0.48
Stock	Male	7	478	4.28	8.25 ± 0.62
	Female	6	306	2.91	7.81 ± 0.56

[1] Analysis of variance for liver fat content (Snedecor):

Source of variation	*Sum of squares*	*Degrees of freedom*	*Variance*
Total	574.48	72	7.98
Diets	174.70	2	87.35 [2]
Sexes	110.67	1	110.67 [2]
Interaction	65.88	2	32.94
Error	223.13	67	3.33

[2] The variances between diets and between sexes are greater than the variance for experimental error by highly significant amounts.

[3] Standard error of the mean.

TABLE 5

Post-mortem frequency of diseases and abnormalities

DIET	SEX	NO. OF RATS	PNEUMONITIS	MIDDLE EAR DISEASE	GENERAL INFECTIONS	HEMATURIA	EDEMA	PARALYSIS	TUMORS		
									Mammary	Pigmented and	Wartlike on tail and feet
Fat	Male	58	52	0	3	3	0	0	3	4	6
	Female	51	37	2	2	1	3	1	18	3	4
Carbohydrate	Male	57	51	1	3	6	0	8	1	5	10
	Female	51	37	5	1	0	2	1	18	5	1
Stock	Male	32	26	2	2	5	1	2	1	2	2
	Female	29	19	1	1	0	0	3	6	3	3

hydrate groups, however, revealed nothing that could be interpreted as a fat-induced cause of premature death.

It is instructive to compare this finding with that of McCay and associates ('35, '43) and Carlson and Hoelzel ('47), who found that an increase in life span resulted from severe caloric restriction and retarded growth. The data presented in figure 1 and table 3 do not support the contention that increased caloric intake per se causes a decrease in the life span, for the animals receiving the carbohydrate diet consumed more calories than the high fat group yet their life span was longer. The efficiency of utilization of the carbohydrate diet, however, was lower than that of the fat diet, for less of the energy intake of the carbohydrate diet was utilized for growth. As noted in other longevity studies (McCay, '49), females on each of the diets lived somewhat longer than males, and no alteration in life span was observed in animals that reproduced and raised a litter of young to weanling age.

The determination of liver fat, shown in table 4, demonstrated a significantly increased liver fat content in males fed the fat diet when compared to males in the carbohydrate group. Histological study of the liver and kidney tissue, however, revealed no evidence of abnormality. A definite sex difference in liver fat content was also apparent, with the males on each diet exceeding the females in this respect.

The post-mortem data (table 5) showed no increased frequency of diseases or abnormalities in animals receiving the fat diet as compared to those in the carbohydrate group. The incidences found agree very well with the data of Saxton ('45, '49) and, in agreement with his statement that "restriction of diet thus increased the life-span without conspicuously altering the cause of death," the present data indicate that a high fat diet decreased the life span without noticeably altering the cause of death. The most common disease recorded was chronic pneumonia with bronchiectasis (described by Ratcliffe, '42), which was found in about 90% of the male and 70% of the female rats on all diets. Hematuria and general

infections were more prevalent in the males, while mammary gland tumors were found more frequently in the females.

SUMMARY

Male and female rats receiving a high fat diet (22.7% fat) grew fully as rapidly as those on a carbohydrate diet (3.4% fat), although they consumed 4.8 and 5.7% less total calories, respectively. No seasonal variation in food consumption occurred on any of the diets fed.

The life span of male rats ingesting the high fat diet decreased markedly and there was also a less pronounced but significant decrease in the longevity of the females. Increased efficiency of utilization of the diet was correlated with decrease in life span, but increased caloric intake per se was not so associated.

The livers of males on the high fat diet contained significantly more fat than those of males fed the carbohydrate diet. The histopathological data and the frequency of diseases and abnormalities, however, revealed nothing that could be interpreted as a fat-induced cause of premature death.

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DEVELOPMENT OF A SEMI-SYNTHETIC DIET FOR YOUNG GUINEA PIGS

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(Received for publication June 13, 1953)

The guinea pig is uniquely valuable for nutritional research because it, like the primates, requires a dietary source of vitamin C. However, it has been used comparatively little for such purposes because of failure to attain a satisfactory rate of growth in early studies with simplified diets. It is also true that the growth rates obtained with the control diets employed in much of this work were unsatisfactory as judged by present standards (Glimstedt, '36; Cannon et al., '45; Booth et al., '49; Roine et al., '49).

In the first attempts to rear guinea pigs on simplified rations, nonpurified ingredients such as yeast were used to supply the B vitamins (Hogan and Ritchie, '34; Davis, Maynard and McCay, '38; Cannon and Emerson, '39; Clark, '41; Hogan and Hamilton, '42). Later, pure vitamins were employed but generally with resulting growth rates of not more than 4 gm per day (Woolley, '42; Woolley and Sprince, '45; Cannon et al., '46). An impetus to the study of guinea pig nutrition was afforded by the demonstration of Booth, Elvehjem and Hart ('49) of the importance of bulk and of certain minerals in the diet. With 15% gum arabic added to a simplified diet in place of an equal amount of sucrose, the average gain per day was 5.1 gm as compared with a gain of only 1.8 gm without the bulk-forming constituent. When the simplified basal ration was supplemented with 25% alfalfa leaf meal, a gain

of 6.5 gm a day was obtained. When ash from alfalfa leaf meal equivalent to 25% of the diet was added to the ration containing gum arabic, a nearly normal rate of growth was obtained. Roine, Booth, Elvehjem and Hart ('49) identified the active components of the alfalfa ash as potassium and magnesium. Since the guinea pig in its natural environment lives on forage, it would normally acquire an adequate supply of these elements. Woodward and McCay ('32) called attention to the accustomed high intake of cellulose and inorganic elements in herbivora.

The best results of Roine et al. were obtained by the addition of 2.5% potassium acetate and 0.5% magnesium oxide to a basal ration containing the following ingredients, in per cent: gum arabic, 15; sucrose, 60.9; casein, 30; salts, 4; soybean oil, 4; and pure forms of all the known vitamins except vitamin B_{12}. This diet permitted an average daily gain in weight of 6.4 to 7.2 gm. This rate of growth was approximately equal to that reported by Glimstedt ('36) for guinea pigs, between 10 and 56 days of age, reared on diets of hay, grain, carrots, rutabagas and fresh grass and was almost equal to the growth rate of 7 to 8 gm per day reported by Cannon et al. ('45) in studies with a commercial pelleted ration containing alfalfa leaf meal, wheat, oats and soybean oil meal.

The purpose of the present investigations has been to devise a diet which could be used in experiments with very young animals, with the aim of obtaining a rate of growth equal to or possibly better than that produced with commercial pelleted diets. Up to the present time there has been little use of very young animals for nutritional studies. Results of previous investigations [1] had indicated that, in general, the younger the guinea pigs the more readily they adapted to experimental diets. Excellent results had been obtained with 10-day-old animals, but since one of the ultimate objectives of the present studies was to determine the quantitative requirement of individual constituents in the diet such as vitamins, it was considered important to remove the young

[1] Unpublished data of senior author.

from the mother at as early an age as possible. Woolley ('42) had previously used animals ranging in age from one to 6 days and Taylor [2] had been successful in rearing a family of three guinea pigs which had been removed from the mother when one day old.

METHODS

Experimental

Animals of the Hartley strain of both sexes, two to 4 days old and ranging in weight from 95 to 115 gm, with an average weight of about 100 gm, were used. At this age there is a good chance of 100% survival. In a preliminary test with 20 animals, removed from the mother when less than 24 hours old, all died, whereas in another test with 19 animals left with the mother from 24 to approximately 32 hours, there were 10 survivors. In most of the experiments herein described a small amount of fresh kale was fed for the first two or three days, especially to those animals under 100 gm in weight. The feeding of kale usually prevented a slight weight loss during this period but did not affect survival.

The experiments herein reported were conducted for 6 weeks unless otherwise stated. Since we feel that the procedures employed have had an important bearing on our success in obtaining uniform development with very young animals, they are presented in some detail, although all of them have not been proved to be essential by controlled experiments. To avoid the danger of their legs' being caught in the screen bottoms of the metal cages, screens of one-fourth-inch mesh were used until the animals were 4 to 5 weeks old; thereafter, one-half-inch mesh screens were employed. Because of the high susceptibility of the guinea pig to infection, often resulting in diarrhea, it seems especially important to follow strict sanitary procedures in rearing the animals. For this reason the drinking equipment was washed and fresh water supplied every day and the cages and racks were cleaned and sterilized once each week.

[2] Unpublished data, Miss D. J. Taylor, National Institutes of Health.

In addition to the regular drinking fountains attached to the outside of the cages, shallow open dishes of water were kept in the cages until the animals learned to drink from the fountains (usually in 7 to 10 days). Glass dishes 60 mm in diameter and 26 mm high (made by cutting the tops from specimen bottles) were found convenient for the purpose. The same type of dish was used for food. The food dishes were placed in holders made of bent sheet metal with an 80 × 64 mm top and a height of 30 mm.[3] A hole in the top, 42 mm in diameter, allowed access to the food. These shallow food dishes are desirable not only because small-sized animals can see the food and have ready access to it, but also because the small amount of food in the dishes is less likely to become stale. When the animals were about two weeks old, larger sized dishes (60 × 60 mm) were substituted.

Although the findings of Booth et al. ('49) and Roine et al. ('49) have been of much value in the present studies on guinea pig nutrition, the diet of the latter investigators has in our hands yielded somewhat variable results, probably because of the particular lot of gum arabic which was used. Some of the animals grew well on the diet, whereas others developed prolapse of the anus, with resulting growth retardation. This difficulty led to the substitution of cellophane spangles for the gum arabic. Additional reasons for the change are that cellophane is more likely to be a uniform product and is less apt to contain contaminating materials. Other investigators had used cellulose in guinea pig diets without adverse effects (Davis et al., '38; Hogan and Hamilton, '42; Woolley and Sprince, '45). Cellophane had also been used in diets as a bulk-forming agent (McCay, '29; Hogan and Ritchie, '33) and Wooley and Sebrell ('45) employed cellophane spangles successfully in their studies on synthetic diets for rabbits. Various other changes were made in the diet, involving substitution of other types of carbohydrates

[3] This type of food container was devised by Dr. Robert Scow of this laboratory.

for part of the sucrose and increasing the content of fat, salts and most of the vitamins, particularly α-tocopherol, niacin, folic acid and ascorbic acid.

The diet (no. 4) employed in preliminary studies contained half the amounts of vitamins shown in table 1 (diet 13) with the exception of folic and ascorbic acids, which were present at levels of 3 mg and 5 gm per kilogram, respectively. In studying ways of improving diet 4, the following variations

TABLE 1

Composition of diet (No. 13)

INGREDIENTS	AMOUNT	INGREDIENTS	AMOUNT
	gm/kg		*mg/kg*
Casein (vitamin-free)[1]	300	Thiamine HCl	16.00
Corn oil	73	Riboflavin	16.00
Sucrose	103	Pyridoxine HCl	16.00
Cellophane spangles[2]	150	Calcium pantothenate	40.00
Starch (corn)	200	Niacin	200.00
Cerelose	78	Biotin	0.60
Potassium acetate	25	Folic acid	10.00
Magnesium oxide	5	Vitamin B_{12}	0.04
Salts (Briggs et al., '52)	60	Vitamin A acetate	6.00
Choline chloride	2	Vitamin D_3	0.04
Ascorbic acid	2	Alpha tocopherol acetate	20.00
Inositol	2	2-Methyl-1,4-naphthoquinone	2.00

[1] An alcohol-extracted product from General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Obtained from the Rayon Processing Company of Pawtucket, Rhode Island.

in the constituents were considered: (a) the level of protein, (b) types of fats, (c) content of cellophane, (d) amounts of the known vitamins, (e) effect of para-aminobenzoic acid (PABA) and (f) addition of aureomycin.

EXPERIMENTAL RESULTS

Results of tests with various modifications of diet 4 are shown in table 2. They suggest strongly that the chief factor limiting the growth of the control groups was an insufficiency of carotene or vitamin A, or both. However, in the

animals receiving the increased amounts of carotene or vitamin A, or both, the incidence of swollen abdomens and rough hair coats was high. The latter conditions were largely overcome by doubling the amounts of all the vitamins except choline and ascorbic acid, but were not associated with a marked increase in over-all weight. No benefit resulted from the addi-

TABLE 2

Effect of variations in some constituents of diet 4

EXPT. NO.	CHANGE IN DIET	NO. ANIMALS	AVE. WT. AT 6 WK.
			gm S.E.[1]
1	No change	6	283 ± 11
	+ 5% alfalfa [2]	6	366 ± 21
	+ carotene (12 mg/kg)	6	343 ± 15
	Butterfat in place of corn oil	6	340 ± 8
	Soybean oil in place of corn oil	6	288 ± 15
	+ 5% cellophane [2]	6	305 ± 22
	With doubled amounts of vitamins except ascorbic acid and choline	6	355 ± 27
	+ aureomycin (50 mg/kg)	6	275 ± 26
2	No change	3	235 ± 20
	Butterfat in place of corn oil	3	289 ± 22
	With doubled amounts of vitamins except ascorbic acid and choline	3	315 ± 13
3	No change	4	255 ± 18
	+ PABA (100 mg/kg)	4	297 ± 22

[1] S.E. determined by method of Mantel ('51).

[2] Additions of 5% alfalfa and 5% cellophane made by replacement of an equal amount of starch.

tion of aureomycin in the amount employed. PABA appeared to be of some value as an addition to this diet. Increasing the cellophane content of the diet by 5% resulted in only a slight increase in growth but a marked spillage of the diet occurred because of its extremely loose, light texture. The use of other types and amounts of bulk-producing materials remains a problem for future investigation.

Further studies were conducted with a new diet (no. 11), using the same amount of choline as in diet 4, half the amount of ascorbic acid and double the amounts of the other vitamins. The results of these experiments are summarized in table 3. It is evident that none of the variations of diet 11 resulted in an improvement in growth. Hence, it would appear that a 30% level of protein in the form of casein is satisfactory for the guinea pig and that the levels of vitamins A and E employed were adequate. The difference in results with butterfat and corn oil is not significant. Further study with PABA should undoubtedly be made, using lower levels than

TABLE 3

Effect of variations in certain constituents of diet 11

EXPT. NO.	CHANGE IN DIET	NO. ANIMALS	AVE. WT. AT 6 WK.
			gm S.E.
1	No change (30% casein)	12	338 ± 13
	Casein reduced[1] to 20%	12	283 ± 11
	Casein reduced[1] to 25%	12	307 ± 15
	+ α-tocopherol (60 mg/kg)	12	338 ± 17
2	No change	6	387 ± 22
	Carotene (12 mg/kg)	6	384 ± 7
	+ PABA (2.5 gm/kg)	6	341 ± 21
	Butterfat in place of corn oil	6	362 ± 22

[1] By replacement with an equal amount of starch.

were employed in these tests. The superiority of diet 11 over diet 4 appears to be in large measure a consequence of doubling the amounts of several of the vitamins. Reduction in the amount of ascorbic acid probably had little effect on the results.

A few changes in diet 11, involving raising the levels of calcium pantothenate and folic acid and lowering the inositol, choline chloride and ascorbic acid levels, were made after further experimentation, and the diet as so modified was designated as diet 13 (table 1). Increasing the amounts of calcium pantothenate and folic acid did not necessarily result in an increase in growth but there tended to be an improve-

ment in the appearance and activity of the animals and possibly a somewhat greater resistance to infection. In general, the amounts of vitamins used in diet 13 were about twice the actual requirement, where known, in order to allow for possible losses which might occur while the diets were standing in the food dishes in the warm animal room (76 to 80°F.).

Results to date indicate that there may be an appreciable difference in the requirement for certain of the vitamins by different strains of guinea pigs. For example, the Hartley strain, a white-furred but not a true albino type which we have used chiefly, appears to have an especially high requirement for folic acid as compared with the Beltsville strain, a slower-growing type of mixed colors. The possibility exists that the higher apparent requirement of the Hartley strain may be related to a tendency of the colony from which the experimental animals were obtained to carry an infection of *Streptococcus hemolyticus*. If these animals were maintained on a diet containing a high level of folic acid (6 to 10 mg/kg), no infection of the gastrointestinal tract was found, but with low levels of this vitamin (1 to 2 mg/kg) a tendency to lymphadenitis of the tract was noted.[4] Studies of this problem are being continued.

Adequacy of diet 13

To test the relative merit of diet 13, the growth rate of animals under the following dietary conditions was studied: (1) diet 13, (2) diet 13 + 5 gm fresh kale daily, (3) pulverized rabbit chow pellets[5] + 0.2% ascorbic acid, and (4) the pellet material + 0.2% ascorbic acid + 5 gm fresh kale daily.

[4] Diagnosis was indicated by histological study but the causal organism was not definitely determined.

[5] Obtained from the Maritime Milling Company of Buffalo, N. Y., and prepared according to specifications of the National Institutes of Health formula with the following ingredients, expressed in per cent: pure oat groats, 15; no. 1 whole wheat, ground, 30.5; alfalfa leaf meal, U. S. no. 1 sun-cured, with carotene content of not less than 60 μg/gm, 40; soybean oil meal, 13; irradiated yeast, 0.25; sodium chloride, iodized, 0.25; calcium carbonate, 1; 8 oz. cold-pressed wheat germ oil per ton of feed.

Table 4 shows the average initial weights of each group of 8 animals, the average at the end of each two-week growth-period, and the average daily gain from the 10th to the 56th day on the diets. There was no significant difference in the appearance of the animals in the different groups. The final weights of those on the pellets supplemented with both ascorbic acid and kale were slightly higher than those of the other three groups but the difference was not statistically significant.

TABLE 4

Comparison of weights of animals (8 per group) fed different diets

DIETS	INITIAL WTS.	AVERAGE WTS. (GM) AFTER SUCCESSIVE 2-WEEK PERIODS				AVERAGE DAILY GAIN FROM 10TH TO 56TH DAYS
		2	4	6	8	
	gm				*S.E.*	*S.E.*
Diet 13	104	169	277	365	458 ± 19	7.1 ± 0.3
Diet 13 + 5 gm kale daily	104	170	268	354	458 ± 18	7.1 ± 0.4
Pellets[1] + 0.2% vit. C	105	181	285	360	454 ± 25	7.0 ± 0.5
Pellets + 0.2% vit. C + 5 gm kale daily	104	180	286	379	478 ± 24	7.5 ± 0.5

[1] Commercial rabbit chow pellets. See footnote 5 of text.

The animals of each of the 4 groups were autopsied when about 9 weeks old. Table 5 shows the length, the weight of the intact body and the weight of the eviscerated carcass of male and female animals. Some of the animals reared on diet 4 in previous experiments had shown a tendency for a considerable accumulation of contents in the gastrointestinal tract, particularly in the cecum, as compared to animals fed the pellets. This tendency was not observed, however, in the animals fed diet 13 in the present test. The following average weights of the tracts minus the esophagus in the 4 groups were as follows: diet 13, 111 ± 2.8 gm; diet 13 + kale, 123 ±

TABLE 5

Physical measurements of guinea pigs after 62 days on diets

(Five male and three female animals per group)

	LENGTH [1] (CM)		WT. — BODY INTACT (GM)		WT. — EVISCERATED CARCASS (GM) [2]	
	Male	Female	Male	Female	Male	Female
Diet 13	10.5 ± 0.18	10.3 ± 0.13	518 ± 26	458 ± 41	408 ± 24	340 ± 38
Diet 13 + fresh kale	10.6 ± 0.10	9.9 ± 0.13	530 ± 16	440 ± 21	400 ± 17	318 ± 10
Pellets + 0.2% vit. C	10.4 ± 0.20	10.0 ± 0.27	521 ± 16	433 ± 26	393 ± 14	326 ± 24
Pellets + 0.2% vit. C + fresh kale	10.1 ± 0.42	9.7 ± 0.23	564 ± 45	462 ± 16	437 ± 32	348 ± 14

[1] Nose to anus.

[2] Entire animal including ezophagus and excluding rest of gastrointestinal tract.

TABLE 6

Organ weights of guinea pigs after 62 days on diets (grams per 100 gm body weight)

	LIVER		KIDNEYS		SPLEEN		ADRENALS		HEART	
	Male (5)	Female (3)	Male	Female	Male	Female	Male	Female	Male	Female
Diet 13	5.97 ± 0.25	6.66 ± 0.87	0.90 ± 0.07	0.86 ± 0.11	0.137 ± 0.008	0.181 ± 0.033	0.046 ± 0.005	0.063 ± 0.002	0.29 ± 0.02	0.34 ± 0.02
Diet 13 + fresh kale	6.87 ± 0.21	7.45 ± 0.75	0.87 ± 0.03	0.85 ± 0.03	0.139 ± 0.013	0.173 ± 0.025	0.049 ± 0.004	0.061 ± 0.002	0.29 ± 0.02	0.34 ± 0.02
Pellets + 0.2% vit. C	7.04 ± 0.25	6.44 ± 0.39	0.99 ± 0.05	0.96 ± 0.19	0.111 ± 0.010	0.161 ± 0.035	0.046 ± 0.002	0.060 ± 0.007	0.32 ± 0.01	0.34 ± 0.02
Pellets + 0.2% vit. C + fresh kale	5.87 ± 0.50	6.30 ± 0.39	0.95 ± 0.05	0.87 ± 0.03	0.113 ± 0.011	0.143 ± 0.015	0.044 ± 0.004	0.054 ± 0.004	0.33 ± 0.02	0.33 ± 0.01

3.6 gm; pellets + vitamin C, 116 ± 3.7 gm; pellets + vitamin C + kale, 119 ± 5.4 gm.

The weights of the livers, kidneys, spleens, adrenals and hearts, expressed as grams per 100 gm body weight, are shown in table 6. These values are given since organ weights of the Hartley strain have not previously been reported. The larger size of the spleens of the female animals is in agreement with Eaton's findings ('38) but the tendency of the adrenals of the females also to be larger than those of the males is contrary to Eaton's observations.

TABLE 7

Average values and standard errors obtained in blood study of guinea pigs reared on different diets

	HEMATOCRIT	HEMOGLOBIN	TOTAL LEUKOCYTES	POLYMORPHONUCLEARS
	%	*gm/100 ml*		
Diet 13	43.4 ± 0.37	14.7 ± 0.20	6,106 ± 506	2,112 ± 200
Diet 13 + kale	43.5 ± 0.27	14.9 ± 0.20	4,062 ± 306	1,356 ± 106
Pellets + 0.2% vit. C	43.2 ± 1.04	15.3 ± 0.50	4,862 ± 250	1,537 ± 200
Pellets + 0.2% vit. C + kale	43.0 ± 0.54	15.6 ± 0.52	4,269 ± 456	1,094 ± 156

The results of blood studies are shown in table 7. Again there was a surprising similarity in the average values obtained for the different groups. The hematocrit values were practically identical. There appeared to be a slight difference in the hemoglobin values for the groups on diet 13 as compared to those on pellets. Measurements of cell volume were not made, so the question of possible differences in the size of the red cells cannot be answered at the present time. The slight variations in total leukocytes and polymorphonuclears are considered not to be significant. The hemoglobin values here found were slightly higher and the total leukocytes slightly lower than those reported for animals of about the same age reared on a diet of natural foodstuffs by Cannon et al. ('45).

Adequacy of the diet for reproduction

Diet 13, herein described, allows reproduction to occur but present results indicate that it is not as satisfactory for the performance of this function as it is for growth. Animals kept on it to an age of 150 days have remained in good health and 5 pairs of animals have been maintained on it through pregnancy and, in each case, litters have been born; some of the young have died but 7 survivors (14 born) seem to be normal in size, activity and general appearance. At 18 days of age one of these young animals weighed 224 gm and the other 236 gm. The mothers were removed from the cages when the young were 4 days old. The young will be continued on the diet to see if another life cycle can be completed.

DISCUSSION

A semi-synthetic diet for guinea pigs has been developed which affords a rate of growth and over-all development comparable to that obtained with a good commercial pelleted diet. Since the diet can be used in routine experiments with reproducible results, it will be possible to study the interrelations of ascorbic acid with other essential nutrients, and especially with different members of the vitamin B complex, to answer some of the unsolved problems in nutrition. It has been used successfully to produce clear-cut deficiencies of thiamine, riboflavin, folic acid, choline, pantothenic acid, pyridoxine and niacin and in evaluating requirements for several of the B vitamins (Reid, '52, '53, and unpublished data). Although, as judged by present standards, the diet appears to be adequate, it is quite possible that further improvements can be made. As pointed out by Mannering ('49), it would be unwise, in view of the present incomplete state of knowledge of the nutrition of the guinea pig, to designate an absolute value for its maximum growth. If unidentified factors are essential, and if such factors are found in kale, they must also be present in the semi-synthetic diet, since the animals appeared to thrive fully as well without, as with, supplements

of fresh kale. Unidentified factors could presumably be present in the casein, cornstarch or corn oil.

SUMMARY

A semi-synthetic diet containing neither liver nor yeast has been developed for guinea pigs from comparatively simple materials. It differs from previously used diets in having a greater variety of carbohydrates and a higher content of both vitamins and minerals. It has supported a rate of growth almost, if not quite, equal to that obtained with a commercial pelleted diet in animals placed on it at two to 4 days of age and continued on it up to 150 days. Animals reared on it seemed to be normal with respect to weight, general appearance, physical activity, appearance and weight of internal organs, hematocrit values, granulocytes and total leukocytes. The diet supports reproduction but further studies are necessary before drawing conclusions as to its value with respect to this function.

ACKNOWLEDGMENTS

We wish to thank Mrs. Ligia Ortiz for her help in the preparation of the diets used in these studies, Miss Mary A. Gray for the blood studies, Dr. Robert Habermann and Fletcher Williams for the histological studies, and Mrs. Clara E. Mowry, Mrs. Mary K. Stull, and W. W. Duvall for assistance in the care of the animals.

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VARIATION OF GROWTH IN SUCCESSIVE EXPERIMENTS WITH FOLIC ACID-DEFICIENT CHICKS AND THE INFLUENCE OF AUREOMYCIN [1]

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(Received for publication July 9, 1953)

Several investigators have reported a sparing action of antibiotics in folic acid deficiency. Biely and March ('51) reported that aureomycin had a folic acid-sparing effect for New Hampshire male chicks fed a corn-casein diet. Other vitamins were also spared by aureomycin. In addition, Coates, Dickinson, Harrison and Kon ('51) reported a slight sparing action of penicillin in folic acid-deficient chicks as well as in chicks deficient in other vitamins. Blaylock, Richardson and Lyman ('52) reported similar results in chicks fed penicillin or aureomycin, although details of their work were not given. In studies with rats made folic acid-deficient by the use of aminopterin, it was found that aureomycin counteracted the effect of the antagonist (Waisman, Green, Cravioto-Munoz, Ramenchik and Richmond, '51).

The results presented here show that dietary aureomycin may depress the growth of folic acid-deficient chicks under certain conditions, contrary to the reports of others. The results also demonstrate that the severity of a folic acid deficiency may be influenced by factors other than the composition of the diet.

[1] Part of these data were presented at the American Institute of Nutrition meeting, April 6 to 10, 1953 (Fed. Proc., *13:* 410, 1953).

EXPERIMENTAL

Day-old female New Hampshire chicks were divided into uniform groups of 6 and raised in electrically heated metal batteries with one-half-inch mesh wire floors. Feed and tap water were given ad libitum and 24-hour uniform lighting was used. Each experiment was conducted for 4 weeks. The chicks used in experiments 1 through 8 and in experiment 13 were obtained from a single breeding flock fed a breeding ration of the same formula throughout. All other chicks were obtained from another commercial hatchery from hens fed a commercial breeding ration.

The composition of the synthetic-type basal diet has been given previously (Briggs, Spivey, Keresztesy and Silverman, '52). The diet was composed of glucose, vitamin-free casein, gelatin, methionine, corn oil, minerals and all identified vitamins known to be required by the chick except folic acid. Crystalline vitamins, including pteroylglutamic acid (PGA), and crystalline aureomycin HCl were used throughout the experiments where indicated.

Because of the possible relationship of the environmental microbial flora to the results, it is important to mention that previous to August, 1951, chicks had not been raised in the present battery room. Since that time the room has been used continuously for chick nutrition experiments. No signs of an infectious disease have been apparent throughout this time. The water pans were cleaned once each 24 hours and fresh water was given daily. At the end of each experiment the batteries were carefully cleaned and all removable parts were steam sterilized. Not all parts of the batteries in contact with the chicks could be steam sterilized, however.

RESULTS

The principal results are shown in table 1. During the first experiments only a mild folic acid deficiency was obtained in the birds fed the basal diet, as indicated by the reasonably good growth obtained as well as by the good appearance of the feathers. In experiment 7 and in experiments 9 through

14 typical deficiency signs and poor growth were obtained. In experiment 13, in which chicks from the same source as in experiments 1 through 8 were used, three of the 6 chicks died with severe folic acid deficiency signs and the three remaining birds had very poor feathers as well as poor growth.

TABLE 1

Four-week weights of folic acid-deficient chicks at various time intervals and the influence of aureomycin
(6 chicks started per group)

EXPERIMENT NO. AND DATE	AVE. WT. IN GM. STANDARD ERROR,[1] AND NO. CHICKS (IN PARENTHESES) SURVIVING AT 4 WEEKS			
	No PGA in basal diet		3 mg PGA/kg of diet	
	No aureomycin	50 mg of aureomycin/kg	No aureomycin	50 mg of aureomycin/kg
1. 9– 4–'51	280 ± 45.2 (4)	184 ± 19.3 (4)	351 ± 18.3 (6)	.....
2. 10–23–'51	240 ± 19.0 (6)	98 ± 8.6 (5)	370 ± 38.4 (6)	.....
	205 ± 10.8 (6)	.....	372 ± 13.3 (6)	.....
3. 12– 4–'51	254 ± 21.0 (6)	131 ± 17.0 (4)	411 ± 15.3 (6)	.....
4. 1–15–'52	289 ± 16.5 (6)	.....	327 ± 12.6 (6)	.....
5. 2–12–'52	265 ± 14.2 (5)	114 ± 17.2 (6)	371 ± 9.3 (6)	410 ± 22.7 (6)
6. 2–26–'52	263 ± 11.8 (6)	148 ± 18.4 (5)	342 ± 18.3 (6)	368 ± 15.0 (6)
7. 4– 8–'52	155 ± 24.3 (6)	118 ± 10.0 (4)	366 ± 23.7 (6)	379 ± 23.3 (6)
8. 5–20–'52	239 ± 36.4 (5)	128 ± 26.0 (4)	363 ± 26.0 (5)	.....
9. 8–12–'52	153 ± 27.4 (5)	126 ± 11.8 (4)	353 ± 16.0 (6)	365 ± 23.3 (6)
10. 9–23–'52	.....	126 ± 17.2 (5)	349 ± 20.7 (6)	353 ± 17.2 (6)
11. 12–30–'52	105 ± 10.0 (3)	98 (1)	328 ± 15.7 (6)	.....
12. 2–10–'53	179 ± 11.0 (6)	138 ± 7.5 (4)	343 ± 10.7 (6)	339 ± 16.8 (6)
13. 2–24–'53[2]	168 ± 35.7 (3)	171 ± 19.3 (6)	388 ± 22.0 (5)	393 ± 24.3 (6)
14. 2–24–'53	167 ± 21.5 (6)	148 ± 11.2 (6)	341 ± 38.3 (6)	372 ± 46.2 (6)

[1] Standard error determined by method of Mantel ('51).

[2] Chicks used in experiment 13 from same source as those used in experiments 1 through 8.

In the early experiments (experiments 1 through 6 and 8) the feeding of aureomycin in the absence of folic acid resulted in the appearance of the usual folic acid deficiency signs, including poor growth and poor feathering, contrary to the results with the basal diet. In the later experiments, in which chicks fed the basal diet grew slowly, the presence or absence of aureomycin had little or no influence on growth or on the

signs of folic acid deficiency. The chicks fed aureomycin showed no great change in severity of symptoms over the course of the experiments. It is important to note that in none of the 12 experiments did aureomycin increase the growth of the folic acid-deficient chicks, contrary to the experience of other investigators.

Normal growth was obtained when ample PGA was added to the diet (column 4, table 1). The further addition of aureomycin (column 5) resulted in a very slight increase in growth in most of the experiments, as would be expected in view of the well-known growth-promoting action of antibiotics (see review by Mickelsen, '53), but the differences were only of

TABLE 2

Level of aureomycin necessary to retard growth of folic acid-deficient chicks
(6 chicks started per group)

MG AUREOMYCIN PER KG OF BASAL DIET (No folic acid)	AVE. WT. IN GM, STANDARD ERROR, AND NO. CHICKS SURVIVING AT 4 WEEKS	
	Expt. no. 3	Expt. no. 5
None	254 ± 21.0 (6)	265 ± 14.2 (5)
10	.....	118 ± 17.0 (4)
20	98 ± 10.5 (4)	103 ± 19.2 (4)
50	131 ± 17.0 (4)	114 ± 17.2 (6)
100	141 ± 8.8 (6)	137 ± 18.0 (6)

borderline significance. No depressing action of aureomycin was noted when it was added to the complete diet, contrary to the results with the deficient diet.

Studies on effective levels of aureomycin

Two studies were made with aureomycin during the early experiments to establish, if possible, the minimum effective level which might cause growth depression. The results are presented in table 2. Although definite conclusions as to minimum levels cannot be reached, it appears that as low a level as 10 mg of aureomycin per kilogram of diet may depress growth significantly when the basal group of animals is high in weight. No amount of aureomycin tested, up to 500 mg

per kilogram of diet, in several of the recent experiments had any depressing effect on growth (data not shown).

Other observations

Several trials were conducted with other antibiotics and chemotherapeutic compounds. It was found that in those early experiments in which aureomycin caused growth depression, a similar effect was obtained with 50 mg of terramycin per kilogram of diet (one trial), 50 mg of chloromycetin (two trials) and 0.5% of succinylsulfathiazole (two trials). In the same experiments 50 mg of procaine penicillin G per kilogram (three trials) and of bacitracin (one trial) showed no

TABLE 3

Aureomycin effect and level of folic acid in experiment 12, 2–10–'53
(6 chicks started per group)

LEVEL OF PGA/KG OF DIET	AVE. WT. IN GM, STANDARD ERROR, AND NO. CHICKS SURVIVING AT 4 WEEKS	
	No aureomycin	50 mg of aureomycin/kg
None	179 ± 11.0 (6)	138 ± 7.5 (4)
0.1 mg	252 ± 10.3 (6)	244 ± 10.3 (6)
3.0 mg	343 ± 10.7 (6)	339 ± 16.8 (6)

effect on growth. Further work is necessary along these lines before final conclusions can be drawn.

It was important to know whether or not aureomycin would depress growth in the later experiments when just enough PGA was present to bring growth up to 200 to 300 gm. The data presented in table 3 show that aureomycin did not depress growth when 0.1 mg of PGA per kilogram was present in the diet. It appears that in the later experiments aureomycin had no effect on the folic acid requirement.

DISCUSSION

Perhaps the most interesting aspect of the results is the relatively mild folic acid deficiency produced in our "new" quarters during experiments 1 through 6 and 8, followed by

a variable but definite increase in the severity of the deficiency in later experiments. In the presence of aureomycin, however, a typical deficiency was produced throughout the period and there was less variation of results from experiment to experiment.

The reason for this change in severity of symptoms without a change in diet is not known. The differences are obviously greater than expected normal biological variation. There are many possible reasons for the differences but it is considered most likely that the cause was indirect, involving intestinal or environmental flora, or both. The growth-depressing action of oral aureomycin but not of injected aureomycin (three injections weekly of equivalent levels in 6 birds over a 4-week period; unpublished data) during the early experiments supports this hypothesis. Further support is found in the similar depressive action of orally administered succinylsulfathiazole and choloromycetin, which are structurally unrelated. Although several different batches of casein, gelatin and glucose were used, of necessity, in the experiments, it is well known that these products are very low in folic acid. Because of this, and because aureomycin caused growth depression in the early experiments, it is felt that our results were not affected by the folic acid content of the dietary ingredients. Although conclusive proof cannot be offered, the authors believe that possible differences in the folic acid stores in the chicks from two sources did not influence the results, since in experiments 7 and 13, which had chicks from the same source as those used in experiments 1 through 6 and 8, poor growth and typical folic acid deficiency signs were obtained. Also, evidence for the similarity in stores of folic acid in the chicks from the two sources is indicated by the reasonable uniformity of growth of the chicks fed aureomycin throughout the experiments. It is possible, however, that differences other than folic acid stores, such as the intestinal microflora, for example, in the day-old chicks from the two sources could have been a factor in the results obtained. Such postulated differences

are of the type one might find even with animals from the same source obtained over a period of time.

The growth of the deficient birds in the early experiments was better than that reported by others who have used similar diets, such as in studies with New Hampshire chicks by Lillie and Briggs ('47) and Franklin, Stokstad and Jukes ('47), and in studies with other breeds of chicks by Robertson et al. ('46), Hill and Briggs ('51), Coates, Kon and Shepheard ('50), Luckey, Moore, Elvehjem and Hart ('46a, b), and others. Thus, only toward the later part of our experiments (experiments 7 and 9 through 14) could we consider our conditions typical. It could be postulated that in the early experiments the intestinal flora synthesized more folic acid-active (or sparing) compounds, or destroyed less, than in the more recent experiments. Folic acid synthesis in the drinking water by a different environmental flora in the early experiments is a possible source of folic acid. However, unpublished data obtained with distilled drinking water and with $CuSO_4$-treated drinking water indicate that contamination of drinking water was not an important factor in our results.

The data demonstrate that considerable variations between experiments, which are sometimes obtained between various laboratories and even within the same laboratory, may occur in a nutritional deficiency even when similar diets are fed. This observation is somewhat reminiscent of, and possibly related to, the reported variation in antibiotic-fed animals under equal dietary conditions (Davis and Briggs, '51; Coates, Dickinson, Harrison, Kon, Porter, Cummins and Cuthbertson, '52; and others as reviewed by Mickelsen, '53).

No direct explanation, other than differences in experimental conditions and intestinal flora, can be offered for the apparent disagreement of our results with those of others who have reported a beneficial effect of antibiotics in folic acid-deficient animals. It may be important to note that we used female chicks in our studies, whereas Biely and March ('51) used male chicks. Other differences in experimental conditions were present, including differences in diets and

source of chicks. The fact that an antibacterial agent such as aureomycin might increase the need for folic acid is not too surprising, since it is well known that certain sulfa drugs may have a similar action in various animal species, including the chick (Briggs, Luckey, Mills, Elvehjem and Hart, '43; Luckey, Moore, Elvehjem and Hart, '46a), the rat (Nielsen and Elvehjem, '42; Daft and Sebrell, '43; Wright and Welch, '44), and the rabbit (Simpson, Schweigert and Pearson, '49). On the other hand, in studies with the chick it has been reported that succinylsulfathiazole had no influence on a folic acid deficiency (Robertson et al., '46; Campbell, Brown, Bird and Emmett, '46).

Since commercial poultry rations contain ample folic acid, it is important to point out that our results should not be construed to show any possible harmful effect of aureomycin when used in commercial poultry rations. In fact, our data confirm the fact that when ample folic acid and other nutrients are present in the ration, aureomycin has no detrimental effect and may even be beneficial, as is well known.

SUMMARY

A study involving 12 successive 4-week experiments was made with female New Hampshire chicks fed a folic acid-deficient diet under as nearly identical conditions as possible. Mild folic acid deficiency signs were produced in the first half of the study, followed by a marked increase in the severity of deficiency signs in the latter half of the study in spite of no change in the diet. Dietary aureomycin markedly depressed the growth of the deficient chicks during the early experiments but had very little effect in later experiments when typical folic acid deficiency signs were produced by feeding the basal diet alone. In no instance did aureomycin increase the growth of the folic acid-deficient chicks. The differences in results were interpreted as possibly being due to changes in environmental or intestinal microflora, or both, over the course of the experiments, although other possibilities exist.

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EFFECTS OF PROLONGED PERCUTANEOUS ADMINISTRATION OF METHYL TESTOSTERONE AND ESTRADIOL ON GROWING FEMALE RATS[1]

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SIXTEEN FIGURES

(Received for publication June 16, 1953)

INTRODUCTION

In order to study the effects of upsetting the balance between male and female sex hormones in the animal body, estradiol and methyl testosterone were given by percutaneous administration to growing male rats (Light and Tornaben, '53). It was found that estradiol severely retarded the growth and development of these male rats, as was found in less lengthy experiments by other investigators (Emmens and Parkes, '47). However, food and water consumption were definitely increased, indicating a much greater physical activity. Methyl testosterone appeared to increase protein deposition and cause enlargement of the kidneys, as previously reported (Kochakian, Robertson and Bartlett, '50). The following presentation is a continuation of the above study, using female rats. The results are compared with those obtained from the male rats, and show that in the animal body the estrogenic hormone limits growth but stimulates food consumption and undoubtedly physical activity, whereas the androgenic hormone sponsors growth, with lessened food intakes and decreased physical activity.

[1] Presented in part at the 17th annual meeting of the American Institute of Nutrition at Chicago, Ill., April, 1953. See Proceedings of the Federation of American Societies for Experimental Biology, 12: part 1, p. 421, 1953.

EXPERIMENTAL PROCEDURES

Recently-weaned female rats[2] were divided into three groups of 5 animals each, placed in oblong cages and supplied with water and an adequate diet of ground fox chow ad libitum. The rats were appropriately inuncted with the sex hormones and various experimental data were collected, as previously described (Light and Tornaben, '53), for 186 days.

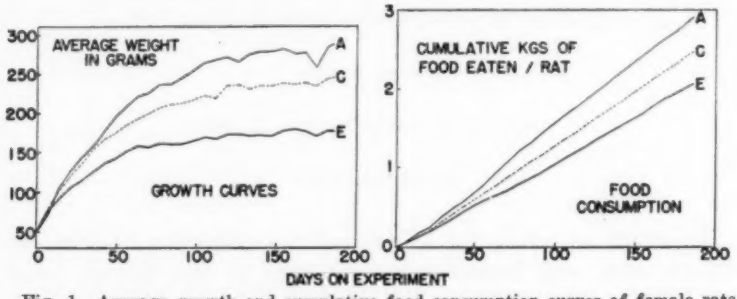

Fig. 1 Average growth and cumulative food consumption curves of female rats inuncted with estradiol (E), methyl testosterone (A) and placebo (C) ointments.

RESULTS

Growth

Methyl testosterone definitely stimulated the rate of growth of the female animals (fig. 1), whereas estradiol inhibited growth to the same extent as in the estrogen-treated male rats. The two dips in the upper part of the growth curve of the androgen-treated animals were due to two different rats losing weight at those particular times, followed by gradual recovery in each rat. During the period of weight losses each animal passed bloody urine, which was undoubtedly caused by renal damage, as is shown by histological studies discussed later.

Food consumption

The actual cumulative food consumption (fig. 1) was greatest for the androgen-treated animals and least for the estro-

[2] Carworth Farms.

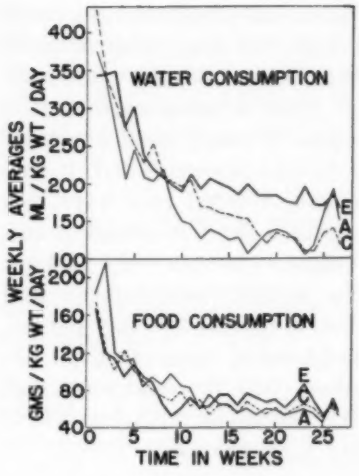

Fig. 2 Food and water consumption expressed in gm and ml per kg body weight.

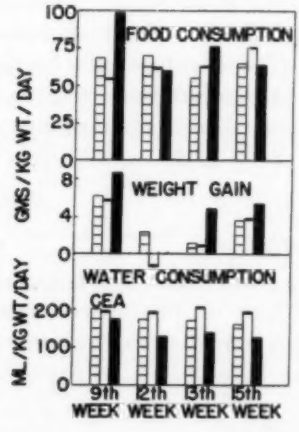

Fig. 3 Comparison of average food and water consumption and weight gain during 4 weekly periods of the growth curve.

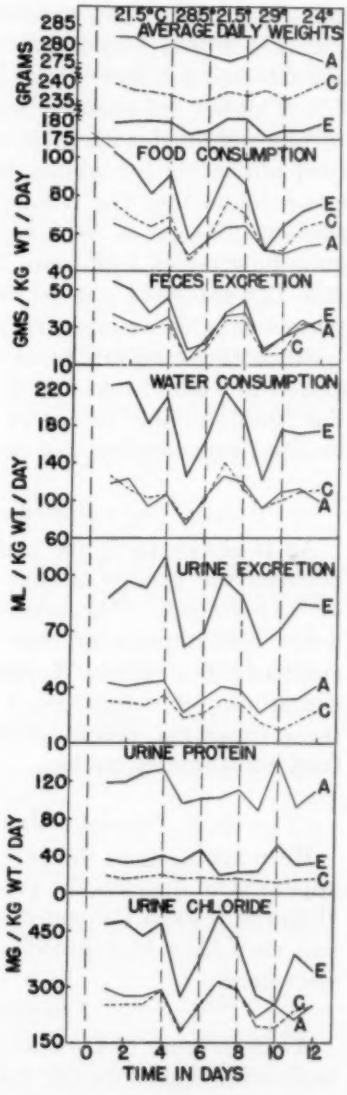

Fig. 4 Temperature effects.

Figures 2–4

gen group. However, when compared on a body weight basis (fig. 2), it was found that although the androgen-inuncted animals had a larger intake at first, the estrogen-treated rats had a higher value subsequently. This is comparable to the results obtained with male animals. When the efficiency of food utilization for adding body weight is considered, it was again found that the female hormone-treated rats were less efficient: 16.4 gm of food were required for the estradiol animals to produce 1 gm gain in weight, whereas only 12.4 gm and 12.8 gm were needed by the methyl testosterone- and placebo-treated animals, respectively, for the 186-day period.

Since the comparable estradiol-treated male animals required 21.3 gm (Light and Tornaben, '53), it would seem that the female is able to accept extra estrogens with less effect on food consumption and activity than the male.

Water consumption

As is shown in figure 2, differences in water consumption between the groups became apparent after about 10 weeks on experiment. The estradiol definitely caused increased water consumption at that time. These values are further emphasized in figure 3, and are compared with food consumption and weight gains for specific weekly periods. At these times the weight gains had very little relationship to food consumption values.

Hair growth

Here again, as in the case of the male rats, both hormones appeared to depress the growth of hair on the clipped areas, although toward the end of the experiment even the controls had very little hair growth. These results will be described elsewhere.

Temperature effects

As in the case of the male rats, the environmental temperatures were varied during the last few days of the experiment. It was found as before that food and water consumption, fecal and urinary excretion, and urinary chloride decreased with

increases in temperature in the range studied (fig. 4). Greatest variations were exhibited by the estrogen-treated animals. The body weights of the animals remained quite constant, which emphasizes the idea that lowered temperature favors increased food consumption accompanied by increased ac-

TABLE 1

Summary of autopsy weights and measurements

	ESTRADIOL	METHYL TESTOSTERONE	CONTROLS
Weight, gm per rat	177.0[1,2] ± 4.3 [3]	288.0[2] ± 21.0 [3]	245.6 ± 8.7 [3]
Body length (minus tail) cm per gm rat	0.102[1,2] ± 0.002	0.073[1] ± 0.003	0.083 ± 0.001
Tail length, cm per gm rat	0.090[1,2] ± 0.003	0.066[1] ± 0.004	0.075 ± 0.003
Specific gravity of carcass	1.103[1,2] ± 0.014	1.081[1] ± 0.010	1.063 ± 0.014
Pituitary, % body weight	0.0140[1,2] ± 0.005	0.0035 ± 0.002	0.0051 ± 0.001
Adrenal, % body weight	0.0149[2] ± 0.0013	0.0081[1] ± 0.0014	0.0141 ± 0.0022
Ovary, % body weight	0.0104[1] ± 0.0031	0.0114[1] ± 0.0018	0.0183 ± 0.0029
Uterus, % body weight	0.308[2] ± 0.011	0.216 ± 0.021	0.259 ± 0.054
Kidney, % body weight	0.469[1,2] ± 0.040	0.555[1] ± 0.109	0.412 ± 0.028
Gastrocnemius muscle, % body weight	0.231 ± 0.022	0.191 ± 0.030	0.212 ± 0.024
Femur bone, % body weight	0.388[1,2] ± 0.018	0.335 ± 0.023	0.335 ± 0.020
G.I. tract and contents, % body weight	11.301[2] ± 0.574	9.827 ± 0.989	10.031 ± 1.230
Submaxillary salivary gland, % body weight	0.108 ± 0.006	0.106 ± 0.010	0.129 ± 0.057
Thyroids and tracheal ring, % body weight	0.0250 ± 0.0030	0.0225 ± 0.0020	0.0239 ± 0.0021
Heart, lungs and thymus, % body weight	1.743[1,2] ± 0.170	1.471 ± 0.161	1.482 ± 0.107
Spleen, % body weight	0.219[2] ± 0.036	0.170 ± 0.013	0.179 ± 0.028
Liver, % body weight	4.236 ± 0.213	4.684[1] ± 0.407	3.978 ± 0.225

[1] Significantly different from controls at P = 0.05 level using the "t" test.
[2] Significantly different from androgen-treated animals by same test.
[3] Standard deviation.

tivity. Methyl testosterone steps up the output of protein in the urine of these female rats as it did in the males.

Autopsy weights and measurements

The values listed in table 1 indicate that methyl testosterone stimulates growth in female rats, whereas excess estrogen depresses development in a manner identical to its action on males. Estrogen also magnified the ratio of skeletal development to body weight and markedly enlarged the pituitaries. Specific gravity values suggest most fat deposition in the controls, with least in the estradiol-treated animals.

TABLE 2

Rectal temperature and blood sugar values

GROUP	TEMPERATURE	BLOOD SUGAR
		mg/100 ml blood
Estrogen	99.9°F. ± 0.87 [1]	88.0 ± 5.23 [1]
Androgen	101.4 ± 1.30	94.1 ± 6.73
Controls	101.2 ± 1.10	97.9 ± 1.88

[1] Standard deviation. The averages of the estrogen-treated animals are significantly different from the others at P = 0.05 level by the "t" test.

The thyroid weights were high in all groups but methyl testosterone definitely depressed the adrenal growth, although not to the extent found in the male animals. However, the male hormone increased the liver and kidney percentage weights. As in the male animals, estrogen caused a more complete filling of the gastrointestinal tract, emphasizing greater food consumption for these rats.

Wound healing

As in the male rats (Light and Tornaben, '53), estradiol retarded healing of epidermal biopsy cuts in the females, whereas methyl testosterone apparently caused some acceleration of healing.

Rectal temperatures and blood sugar

Preliminary investigation of the rectal temperatures (table 2) revealed that the average values for the estrogen-treated

animals were lower than those for the other two groups. Likewise the average blood sugar value of the estrogen-treated group was lower.

Hematology

Results from the hematological study (table 3) disclosed that estrogen inunction lowered the numbers of both red and

TABLE 3

Summary of hematology study

GROUP	GROUP AVERAGES		
	Hemoglobin	Erythrocytes	Leukocytes
	gm/100 ml blood	*millions/mm³*	*per mm³*
Controls	14.28 ± 0.44 [1]	8.68 ± 0.22 [1]	12,060 ± 2,460 [1]
Estrogen	12.55 [2]± 0.38	6.65 [2]± 0.51	9,125 [2]± 780
Androgen	14.46 ± 0.26	8.46 ± 0.38	11,920 ± 1,890

Differential counts

GROUP	GROUP AVERAGES IN PER CENT VALUES						
	Polymorph.	Juven.	Total Neutro.	Lympho.	Mono.	Baso.	Eosin.
Controls	15.2	7.0	22.2	73.6	0.8	0.0	3.4
Estrogen	21.3	10.2	31.5	65.8	1.0	0.0	1.8
Androgen	26.2	6.8	33.0	63.0	0.4	0.0	3.6

[1] Standard deviation.

[2] Significantly different from other groups at P = 0.05 level by "t" test.

white cells, accompanied by a drop in hemoglobin concentration. Granulocytes (total neutrophils) were increased, showing bone marrow activity only for these types. Erythroblastic activity was inhibited. This may be summed up by stating that anemia and leukopenia were apparent. The androgen-treated animals exhibited only an increase in the granulocytes.

Histopathology

Histopathological studies were made of various tissues. The spleens of the androgen-treated animals exhibited slightly diffuse lymphatic tissue, with an occasional reaction center.

The zona fasciculata of the adrenals in the estrogen-treated animals was somewhat narrower than the others, with some depletion of lipid in the external layer of cells. The pituitaries of the oreton-inuncted animals exhibited slight hyperemia and intensely stained basophillic nuclei in the epithelial portions, with occasional nuclear changes (plate 1). Those of the estrogen-treated rats had some hyperemia and a few mitotic figures were observed. The thyroids of the androgen-inuncted animals contained grossly hypertrophied follicles, indicating secretion and storage of colloid substance (plate 2). The uteri of the estradiol-treated animals showed transitional stratified squamous epithelium replacing much of the normal columnar epithelium which had partially atrophied with darkly stained and pycnotic nuclei. The ovaries of these animals gave a picture of increased abnormal follicular production but with some germinating follicles present. The androgen-treated animals showed hyperplasia along with hyperfunctional follicular production.

The kidneys of the two androgen-treated rats which caused dips in the growth curve had grossly enlarged tubules, with the epithelium in various stages of deterioration in certain areas. Tubules not undergoing changes contained fine particulate masses, probably hematin, and occasionally hyaline casts. Some glomeruli contained thickened capsular epithelium. The kidneys of the other two groups of rats had no abnormalities. It may be stated that both hormones, in excess, caused certain histological changes in the female rats.

DISCUSSION

When the above data derived from female rats were compared with the results obtained from male rats inuncted with estradiol or methyl testosterone, it became apparent that the male hormone favors growth, whereas the female counterpart fosters activity along with increased food intake. This has also been demonstrated by Kim, Magee and Ivy ('52) and discussed by Light and Tornaben ('53). The pituitary

glands of male animals usually are smaller than those of the females and do not change appreciably with the addition of methyl testosterone, but with the addition of estradiol their enlargement is quite remarkable, as is discussed by Emmens and Parkes ('47). The larger pituitaries in the female are likewise enhanced with estradiol but depressed with methyl testosterone. The adrenals follow the same pattern except that the usually larger adrenals in the female are not greatly magnified by estradiol, whereas they are definitely suppressed in growth by methyl testosterone. Although the estrogen-treated rats were impaired in growth and their carcasses were low in fat content, the increased adrenal development and other differences precluded any similarity with the animals on a fat-free diet described by Panos and Finerty ('53). It has been reported by Clark ('53) that cortisone retards the weight gain of rats having the same food intake as their controls. Cortisone also retarded wound healing, which is similar to present observations (Howes, Plotz, Blunt and Ragan, '50).

The thyroid glands of male rats appear to be enlarged by estrogens, with probable increased secretion and release of the thyroid hormone, whereas the usually larger gland in the female, although not definitely impaired in growth, seems to have less secretory activity following administration of methyl testosterone. This resulted in a decrease in the physical activity of the animals with lowered food consumption but with gain in weight. Excess thyroid definitely retards growth but with increased temperatures (Kennelly and Maynard, '53). The increased liberation of the thyroid hormone following estrogen administration is suggested by the work of Engstrom et al. ('52), who found increased serum-precipitable iodide in both men and women following the administration of estrogen. The weights of the heart, lungs and thymus groups of glands are also enlarged by the estradiol treatment. This would suggest the increased thyroid activity described by Andrus ('53).

In these long-term experiments the growth of the ovaries and testes of rats is likewise impaired in the respective animals by both hormones. The uteri, however, are enlarged by estradiol treatment. Estrogens may also exert lipotropic activity and a beneficial effect in the prevention of dietary cirrhosis (György, '52). In addition, they may increase the percentage weights of the islands of langerhans (Kerr et al., '52). Some additional insulin output may be the reason for the slightly lower blood sugar levels observed in the estrogen-treated animals. On the other hand, the inhibition of thyroid activity brought about by methyl testosterone may accelerate the development of circulatory disorders. In both sexes the kidneys are enlarged following administered androgen and in the female rats definite damage was found in two of the 5 animals. While this number is not conclusive, it may indicate a future pathway in the study of kidney malfunctions and possibly the related hypertensions. The upset of the usual balance between estrogens and androgens has likewise been postulated as decreasing resistance to cancer formation (Nicol and Abou-Zikry, '53).

CONCLUSIONS

1. Growing female rats maintained on an adequate diet but inuncted with estradiol ointment were severely retarded in growth and development to a degree comparable to that shown by male animals similarly treated. However, when considered on a body weight basis, food and water intakes were increased, indicating low efficiency in producing body tissue. Environmental temperature changes caused greater variations of observed values in these estrogen-treated animals.

2. Methyl testosterone, on the other hand, increased the growth of the female rats, possibly by depressing the activity of the pituitary, adrenal and thyroid glands. The efficiency of tissue formation from food consumed was much greater in these animals than in those inuncted with estradiol.

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PLATE 1

EXPLANATION OF FIGURES

5 Ovary (control). × 200.

6 Ovary (estrogen-treated) with an increase of smaller follicular cells with a very compact stroma.

7 Ovary (androgen-treated) with hyperplastic development exhibiting hyperplasia of the granulosa layer, thinning of the theca and stromal activity.

8 Pituitary (control). × 200.

9 Pituitary (estrogen-treated) showing hyperplasia of all elements and with follicularization indicating increased secretion.

10 Pituitary (androgen-treated) showing hyperplasia of chromophobe cells predisposing minimum secretion.

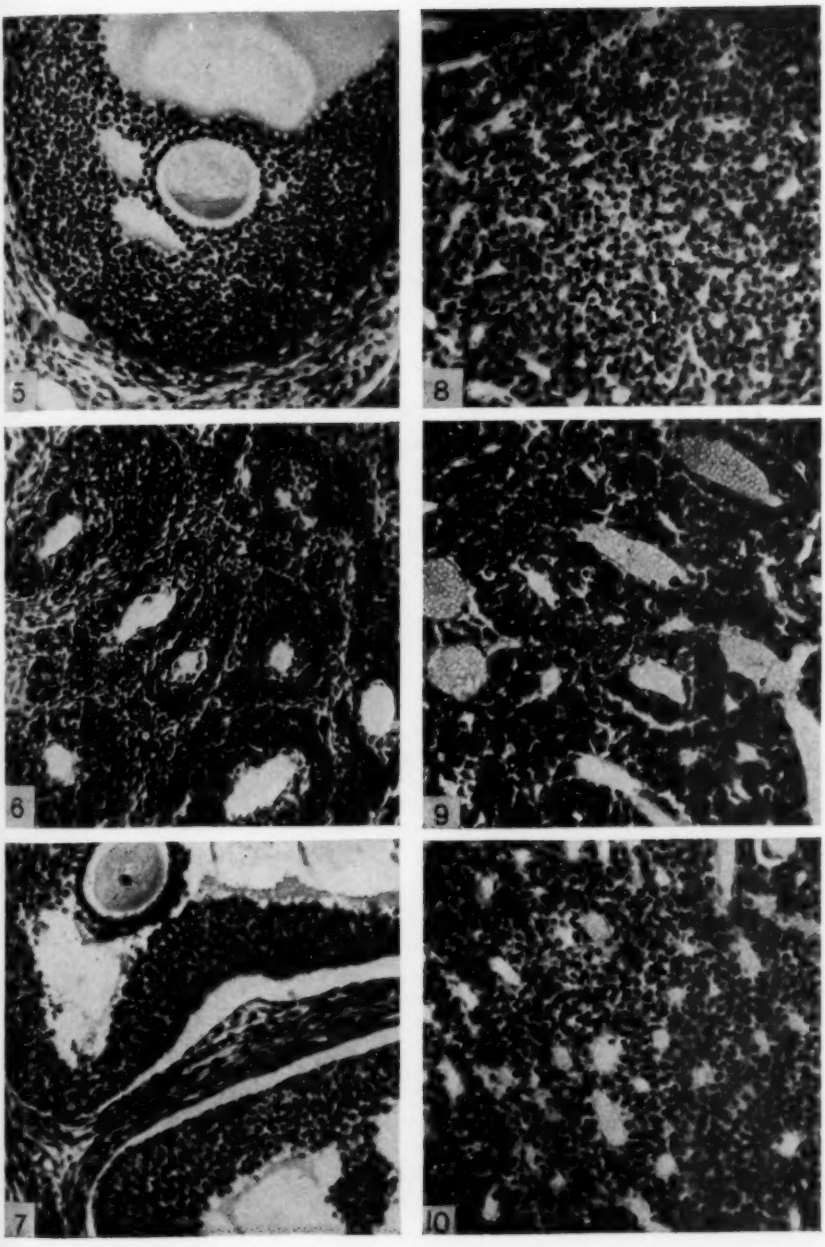
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PLATE 2

EXPLANATION OF FIGURES

11 Thyroid (control). × 200.

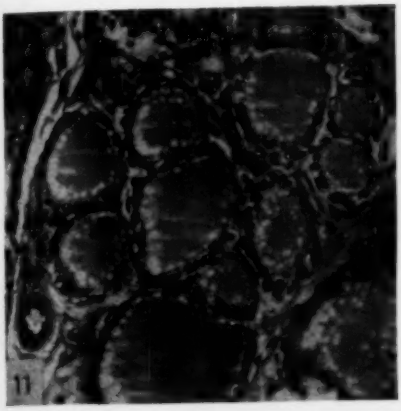
12 Thyroid (androgen-treated) with a grossly hyperplastic follicle surrounded by hypertrophied peripheral secretory cells. × 200.

13 Uterus (control). × 200.

14 Uterus (estrogen-treated) with squamous metaplasia and a stroma showing proliferative changes. × 200.

15 Kidney (control). × 200.

16 Kidney (androgen-treated) with hydropic degeneration and vacuolization of tubular epithelium, especially in the proximal limbs of the tubules. × 200.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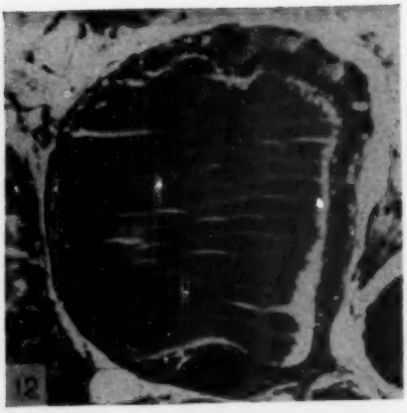
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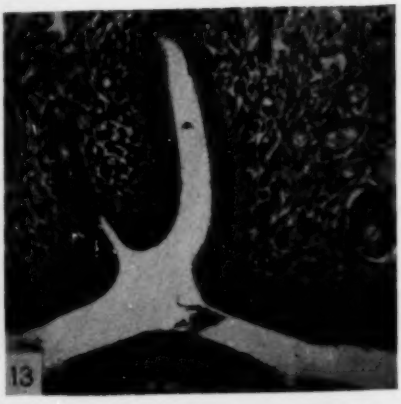
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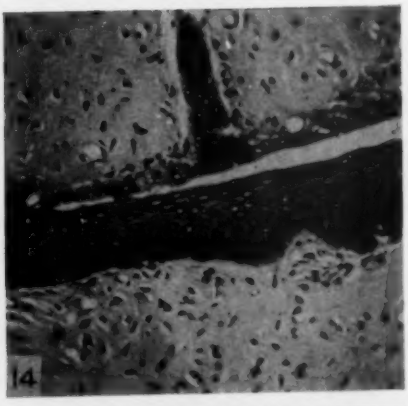
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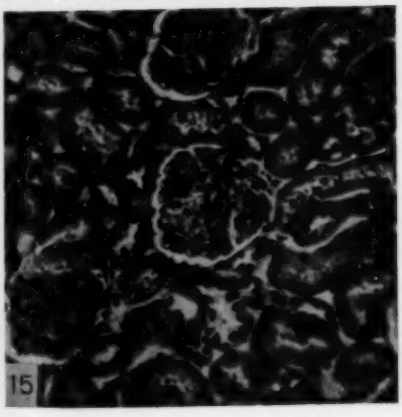
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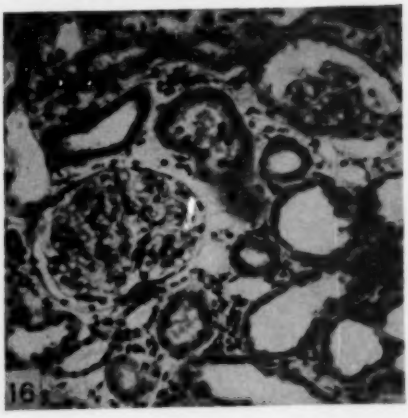
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P^{32} DISTRIBUTION AND EXCRETION IN RATS FED VITAMIN D-FREE AND LOW PHOSPHORUS DIETS[1, 2]

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THREE FIGURES

(Received for publication June 22, 1953)

Studies of the effects of low phosphorus diets and vitamin D deprivation on growth, total phosphorus content of bone and soft tissues, and total phosphorus excretion have been carried out by Bethke, Kick and Wilder ('32), Day and McCollum ('39), Nicolaysen and Jansen ('39), Schneider and Steenbock ('39), Coleman et al. ('50) and Steenbock and Bunkfeldt ('51). Cohn and Greenberg reported on the distribution of radioactive phosphorus^{32} in the tissues of rats on a complete ration ('38) and of rats on rations low in phosphorus content and without vitamin D ('39). Manly and Bale ('39) studied the uptake of this isotope by rat bones and teeth, the animals in these studies having been fed adequate diets. No record has been found in the literature of studies of P^{32} distribution either in growing rats deprived only of vitamin D or in growing rats on a diet very low in phosphorus, supplemented with adequate vitamin D. In the present study the rates of deposition and turnover of P^{32} by the blood, liver,

[1] Florida Agricultural Experiment Station Journal Series 168. Published with the approval of the Director of the Florida Agricultural Experiment Station. This work was supported in part by funds granted by the Nutrition Foundation, Inc.

[2] Presented at the 122nd annual meeting of the American Chemical Society at Atlantic City, New Jersey, September, 1952.

kidney, muscle and bone, and of excretion of P^{32}, were followed in weanling rats fed diets that were (1) adequate, (2) adequate but containing no vitamin D, and (3) inadequate in phosphorus.

EXPERIMENTAL

A basal ration was prepared consisting of 18% protein (egg albumin steamed three hours before use), 5%fat,[3] 73% carbohydrate (sucrose), 4% salt mixture (24.55% $CaCO_3$, 28.89% NaCl, 19.82% $KHCO_3$, 13.23% KCl, 9.37% $MgSO_4$, 3.20% ferric citrate, 0.32% $CuSO_4 \cdot 5H_2O$, 0.10% $MnSO_4 \cdot 2H_2O$, 0.5% $ZnSO_4 \cdot 7H_2O$, and 0.02% KI), and the following vitamins per kilogram of ration: 1,500 U.S.P. units of A, 1.3 mg thiamine, 2.5 mg riboflavin, 1 mg pyridoxine, 10 mg pantothenic acid, 1,000 mg choline hydrochloride, and 1 mg biotin. The calcium content in this basal ration was 0.4% and the phosphorus content 0.014%; thus the calcium-to-phosphorus ratio was 28 to 1.

One hundred and thirty-two weanling rats (24 to 28 days of age) were allotted equally to three dietary groups as follows: (1) control group — basal ration plus 11.13 gm $NaH_2PO_4 \cdot H_2O$ and 3,000 U.S.P. units vitamin D, as viosterol, per kilogram of ration; (2) vitamin D-deprived group — basal ration plus 11.13 gm $NaH_2PO_4 \cdot H_2O$; and (3) low phosphorus group — basal ration plus 3,000 U.S.P. units vitamin D. The phosphorus-supplemented rations contained 0.25% phosphorus, giving a Ca : P ratio of 1.6 to 1, as compared with the 28 : 1 ratio in the low phosphorus diet.

After 10 days on the various diets the rats received 3 μc of P^{32} containing 1 to 3 μg of phosphorus as inorganic phosphate.[4] The isotope was administered intramuscularly to 48 of the rats and orally by stomach tube to the remaining 84.

The animals were sacrificed by ether anesthesia after the following intervals: intramuscular dosage, 12 rats each at

[3] Wesson oil.

[4] The P^{32} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee, after allocation by the Isotope Division of the Atomic Energy Commission.

one-fourth, 5 and 24 hours, three rats each at 4, 8, 14 and 21 days; after oral dosage, 12 rats each at one-fourth, 5 and 24 hours and 4, 8, 14 and 16 days. Equal numbers of rats in each of the three dietary groups were sacrificed at these intervals.

Tissues taken for determination of P^{32} activity and total phosphorus were blood, liver, kidney, gastrocnemius muscle and femur, except that in the intramuscularly-dosed rats sacrificed after intervals of more than 24 hours only blood, liver and bone were taken.

Fecal and urinary excretions were collected up to 14 days after isotope administration and analyzed for P^{32} concentration. Total phosphorus was determined in the feces.

In addition to the weanling rats, 27 rats raised on a stock ration were placed on the adequate, vitamin D-free and low phosphorus diets (9 to each diet) at 100 days of age, and 46 days later were given intramuscular injections of P^{32}. After intervals of 24, 48 and 72 hours these rats were sacrificed and the 5 tissue samples listed above were taken for analysis.

All samples were wet-digested with nitric acid, and P^{32} activity was determined by the use of dipping-type Geiger-Mueller tubes and commercial scalers. Total phosphorus was determined in bone and fecal samples by the volumetric phosphomolybdate method (Association of Official Agricultural Chemists, '50), and in the soft tissues by the molybdenum blue method (Fiske and Subbarow, '25).

Radioautographs of femurs of rats maintained on the three diets for 17 days were made, the rats having been given 7.5 μc of P^{32} by stomach tube 20 hours before sacrifice. The period of exposure of the x-ray film to the bones was 4 hours.

RESULTS AND DISCUSSION

The two groups of rats receiving the diets adequate in phosphorus appeared vigorous throughout the experimental period; the rats on the low phosphorus diet, however, at 18 days began to show progressive weakness of the hind limbs and difficulty in breathing.

Average total phosphorus values for various tissues from 24 rats of each of the three dietary groups maintained on the ration 10 to 11 days are presented in table 1.

A statistical analysis of the total phosphorus values comprising these averages reveals that in none of the tissues studied was there a significant difference between the two adequate phosphorus groups, nor was the difference between the adequate and low phosphorus groups significant in the values determined for the liver, kidney and gastrocnemius muscle. The analysis indicates, however, that in the blood and femur, total phosphorus content was significantly lower in the low phosphorus group than in the adequate phosphorus

TABLE 1

Milligrams of phosphorus per gram of fresh tissue

TISSUE	DIET		
	Control	Vitamin D-free	Low phosphorus
Blood	0.26 ± 0.04	0.26 ± 0.05	0.18 ± 0.04
Liver	2.19 ± 0.53	2.29 ± 0.52	2.32 ± 0.46
Kidney	2.04 ± 0.33	2.14 ± 0.50	2.21 ± 0.44
Muscle	2.37 ± 0.33	2.53 ± 0.55	2.59 ± 0.51
Femur	49.76 ± 12.13	53.63 ± 14.52	37.44 ± 10.61

groups. Thus it appears that in the rats on low phosphorus intake, soft tissue phosphorus was maintained at normal levels by phosphorus withdrawn from the bones. The lowered blood value probably reflects the lowered availability of phosphorus in the bones.

During the period of study, the femurs of the rats receiving the control and vitamin D-free diets remained essentially constant in concentration of phosphorus, while those of the low phosphorus rats decreased from 33 to 15 mg per gram of bone between 11 and 26 days on the ration.

The data obtained for rate of deposition and turnover of P^{32} after oral and intramuscular administration are presented in figures 1 and 2, respectively. Following oral dosage the rate of deposition and turnover of the isotope in the femurs

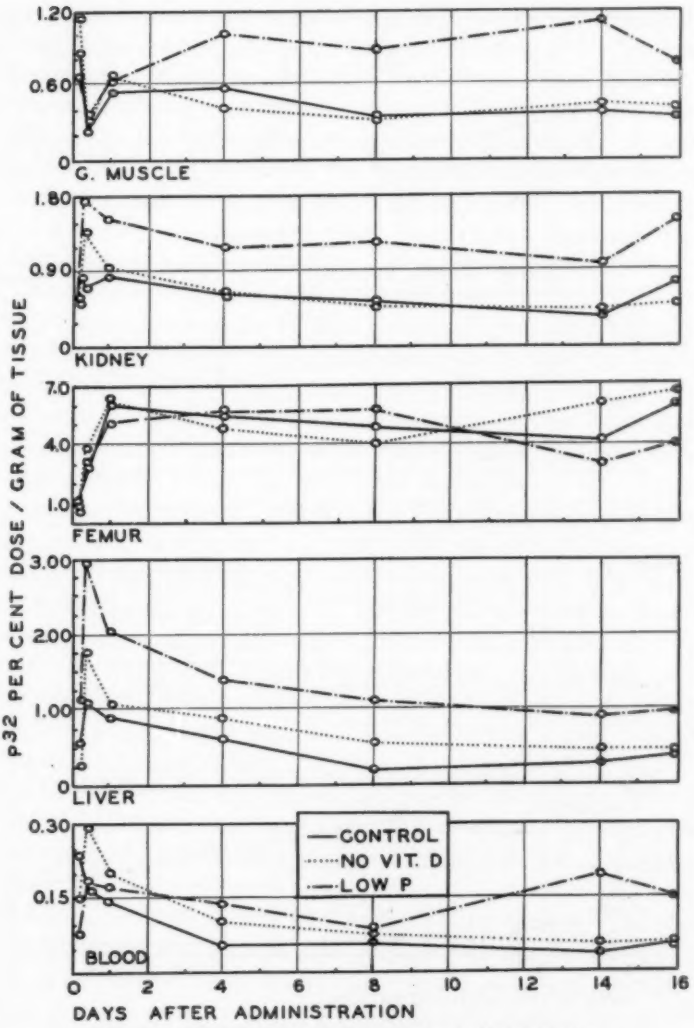

Fig. 1 Distribution of P^{32} following oral administration.

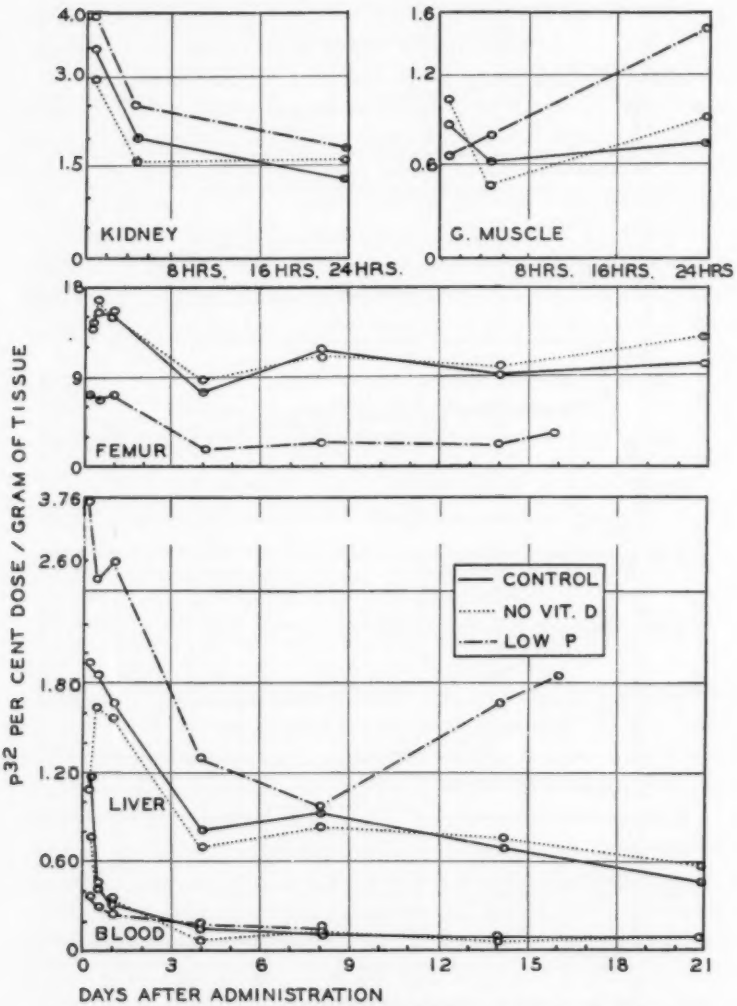

Fig. 2 Distribution of P^{32} following intramuscular administration.

of all three dietary groups followed essentially the same pattern. Intramuscular injection of P^{32} resulted in maximum concentrations in the femur at about 5 hours after dosage; however, in this case, the low phosphorus group showed concentrations only one-third to one-half as great as those found in the femurs of the two adequate-phosphorus groups. The 14- and 16-day values of the intramuscularly-injected low phosphorus rats were obtained from animals which died; no blood samples were secured from these rats. As noted previously, kidney and muscle were not taken from parenterally-dosed rats sacrificed after more than 24 hours.

The tissue showing the highest uptake of P^{32} per gram after both oral and intramuscular administration was the femur, followed in order by liver, kidney, muscle and blood. The low phosphorus group consistently deposited higher concentrations of P^{32} in the soft tissues than did the two adequate phosphorus groups, following both oral and parenteral dosage. Isotope concentrations found after intramuscular injection were two to three times as high as those found after oral administration. Oral dosage required approximately 5 hours for peak deposition in all soft tissues except muscle, which showed its lowest concentration at this time; the femurs required 24 hours for maximum deposition. Following parenteral dosage, maximum soft tissue concentrations were attained in 15 minutes or less, while the femurs required 5 hours to reach maximum values.

Excretion values, expressed as average percentage of administered P^{32} per rat and milligrams of total phosphorus per rat in 14 days following isotope administration, are shown in table 2.

The level of phosphorus in the diet of the control rats was about 18 times that in the low phosphorus diet, while fecal total phosphorus excretion by the control rats was approximately 8 times that of the low phosphorus group. Fecal P^{32} excretion values following oral administration were similar for the two groups. The ratio of P^{32} administered to P^{31} or dietary phosphorus ingested was 18 times as high for the low

phosphorus animals as for the controls (i.e., 3 μc P^{32} to 0.014% P^{31} in the low phosphorus rations, as compared with 3 μc P^{32} to 0.25% P^{31} in the control ration). The ratio of P^{32} to P^{31} in the feces was only about 5 times as great in the low phosphorus rats as in the controls. This might be considered indicative of some degree of retention of the administered P^{32} by the low phosphorus rats, and is in line with the urinary P^{32} excretion data.

TABLE 2

Fourteen-day phosphorus excretion

ROUTE OF ADMINISTRATION		DIET		
		Control	Vitamin D-free	Low phosphorus
			P^{32}, % dose/rat	
Parenteral	Feces	22.4	16.0	15.3
	Urine	15.8	22.9	1.7
Oral	Feces	10.6	17.2	11.3
	Urine	9.9	8.0	0.78
			Total phosphorus mg/rat	
	Feces	60.9	69.1	7.5

In the urine, following both oral and parenteral isotope administration, P^{32} values for the low phosphorus group were approximately one-tenth those found in the control group. This decreased urinary output, noted also by Cohn and Greenberg ('39) in rats on low phosphorus, vitamin D-free diets, is suggestive of a saving of phosphorus through increased renal tubular reabsorption. Total phosphorus was not determined in the urine because of contamination with feed.

The excretion data indicate no effect of vitamin D deprivation on the handling of phosphorus by either the intestine or the kidney.

In the series of rats 146 days of age, the various tissues of the control, vitamin D-deprived, and low phosphorus groups were found not to differ significantly in P^{32} concentration when these animals were sacrificed at 24, 48 and 72

hours after parenteral injection of the isotope. The following ranges of P^{32} concentration were found in the various tissues: blood, 0.2 to 0.4; liver, 1.2 to 2.8; kidney, 1.1 to 1.7; muscle, 0.5 to 1.0 and femur, 2.2 to 3.9% of the dose per gram of fresh tissue. These soft tissue values were slightly higher than, but the femur values only about one-third as high as, those found in the young rats.

The radioautographs obtained from the femurs of rats of the three dietary groups are shown in figure 3. No appreciable difference can be seen in distribution of P^{32} in the control

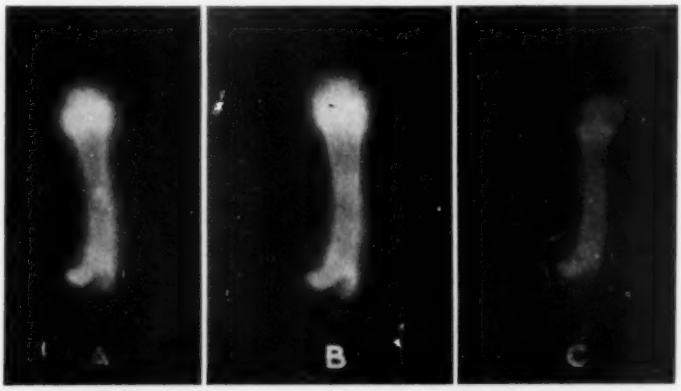

Fig. 3 Radioautographs of femurs: A, control; B, vitamin D-deprived; C, low phosphorus.

and vitamin D-deprived bones. In the case of the femur of the rat fed the low phosphorus diet, P^{32} deposition in the proximal end was negligible, suggesting either a failure in the process of ossification of epiphyseal cartilage, hence a lack of phosphorus for exchange with P^{32}, or somply a lack of exchangeable phosphorus due to loss of this mineral from the bone.

The data obtained in this study on the effects of a low phosphorus diet do not support the work of Schneider and Steenbock ('39), whose findings indicated that in rats fed a diet having a calcium-to-phosphorus ratio of 14:1 and containing

vitamin D, soft tissues were deprived of phosphorus in order to make it available for bones. However, the data are in line with the findings of Day and McCollum ('39), who reported marked skeletal rarefaction in young rats receiving a diet in which the calcium-to-phosphorus ratio was 26 to 1, and those of Coleman and co-workers ('50), who noted the development of severe rickets in rats receiving a diet containing only 0.015% phosphorus, vitamin D being supplied in both the latter diets.

The observation in this study of no appreciable effects of vitamin D deprivation on rats fed a diet in which the calcium-to-phosphorus ratio is optimum, is in agreement with the findings, based on chemical analyses, of Bethke, Kick and Wilder ('32) and Nicolaysen and Jansen ('39), although the former workers found a slight increase in the ash content of the bones upon addition of vitamin D to such a diet. Cohn and Greenberg ('39), studying rats on a low phosphorus, vitamin D-free diet, noted no effect on absorption of phosphorus from the intestine when vitamin D was fed just prior to isotope administration.

SUMMARY

In this study a comparison has been made of the deposition and turnover of orally and intramuscularly administered P^{32} in the blood, liver, kidney, muscle and femur of growing rats maintained on diets that were (1) adequate, (2) adequate but lacking in vitamin D, and (3) low in phosphorus; total phosphorus was determined in the above tissues, and radioautographs were made of the femurs of rats of the three dietary groups.

Essentially no difference was demonstrated by isotope or total phosphorus data between the two groups of rats receiving adequate phosphorus; that is, the absence of vitamin D was without any observed effect in the presence of an optimum calcium-to-phosphorus ratio. On the other hand, the rats of the low phosphorus group deposited more P^{32} in their soft tissues than the rats of the two groups fed adequate phos-

phorus diets, but less in the femur, as indicated by both Geiger counting and radioautographs; total phosphorus values indicated depletion in the femurs but not in the soft tissues. The rats on the low phosphorus diet excreted about the same percentage of isotope in the feces as those on the adequate phosphorus rations, after both oral and parenteral dosage, but showed a marked decrease in the amount excreted in the urine, indicating a considerable capacity in this respect to conserve phosphorus when the intake of this mineral is inadequate. Total phosphorus excretion in the feces of the low phosphorus diet rats was approximately one-ninth that of the adequate phosphorus groups. The radioautographs demonstrated only slight deposition of P^{32} in the proximal end of the femur of the low phosphorus group as compared with the femurs of the rats receiving adequate phosphorus.

ACKNOWLEDGMENT

We wish to thank Winnie R. Teeters, Jon L. Herring, Jason C. Outler and Jay J. Herrmann for technical assistance in this investigation.

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NATURE OF THE "VITAMIN A-LIKE FACTOR" IN LARD

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ONE FIGURE

(Received for publication July 20, 1953)

It has been shown by Kaunitz and Slanetz ('50a) that the occurrence of vitamin A deficiency in rats can be prevented by feeding them a diet containing a molecularly distilled forerun fraction from lard. Their results showed that this fraction in most instances had biological activity equivalent to abount 5 to 25 units of vitamin A acetate per gram. Assuming that this forerun fraction, which amounted to 7% of the lard, contained nearly all the active material, the potency of the original lards would range from about four-tenths to two units per gram. Chemical and spectroscopic analyses by 4 laboratories, however, failed to establish the presence of conventional vitamin A alcohol or ester. Therefore, it was tentatively concluded that the active material belonged to the somewhat vague group of substances possessing biological A activity but assumed to be chemically different from the known vitamin A. Reports of such substances have appeared within the last two decades (Randoin and Netter, '34; Lemley, Brown,

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Bird and Emmett, '47; Dubouloz, Marville and Chevalier, '48; LeGallic, '48; Mayer and Krehl, '48; Grangaud and Massonet, '49; Lane, '50).

The investigation of the nature of this biologically active material was continued. If the material could be concentrated further by molecular distillation, adsorption fractionation, or crystallization, identification might be established. It was also deemed probable that failure to detect vitamin A by the spectroscopic method could be attributed to the presence of unsaponifiable impurities difficult to separate which have significant absorption in the spectral region, 300 to 325 mμ. The difficulties of separating these absorbing impurities in fish liver oils and fortified margarines have been discussed by Gridgeman et al. ('48); Barua and Morton ('49); Morton and Stubbs ('48); Wilkie ('49); and Boldingh and Drost ('51). The difficulties of separating interfering unsaponifiables from the vitamin factor would be expected to be greater for lard than for fish liver oils, butter or fortified margarines, because the factor is present in much lower concentrations and the ratio of unsaponifiables to units of the factor is also likely to be much greater.

The present paper is a report of an extended investigation into the nature of the active factor in lard. Evidence of the presence of the conventional form of vitamin A was obtained.

EXPERIMENTAL

Materials

Two samples [3] obtained as forerun fractions from molecular distillation of two different batches of freshly rendered lard were available from previous work (Kaunitz and Slanetz, '50b). These were distilled at temperatures of up to 215°C. and 3 μ of pressure. The fractions, amounting to about 7% of the lard, had been kept tightly sealed and under refrigeration and still showed biological vitamin A activity. They will be referred to as original distillates (O.D.-I and O.D.-II).

[3] Distillation Products Industries, Division of Eastman Kodak, and Parke-Davis, Inc., kindly furnished the molecular distillate samples.

The biological activity of O.D.-I was equal to 4.5 units of vitamin A per gram; that of O.D.-II to 25 units per gram.

Bioassay technique for determining vitamin A activity

The bioassays were carried out on albino rats. The mothers of the young eventually used were placed, within three days of the births of their litters, on a highly purified, vitamin A-deficient diet. After weaning at 21 days, the young received a more rigidly vitamin A-deficient diet, consisting of 54% cerelose, 30% alcohol-washed casein, 4% salts (U.S.P. No. 2), 2% celluration, 10% rancid lard and, per kilogram, 5 gm $CaCO_3$, 1 gm inositol, 1 gm choline, 300 mg *p*-aminobenzoic acid and 100 mg nicotinic acid. They were fed separately 4 times weekly two drops of a suspension containing, per milliliter, 20 mg calcium pantothenate, 4 mg thiamine, 8 mg riboflavin, 8 mg pyridoxine, 5 mg folic acid, 50 μg biotin, 10 μg vitamin B_{12} and 50 mg ascorbic acid.[4] The lard was made rancid to destroy the vitamin A which it could have contained; there was no objection to this procedure because it has been proved that mildly rancid lard is not toxic (Stoerk, Kaunitz and Slanetz, '52).

When the animals were 28 days old, groups of 12 matching young were made up. The average weights of the groups at 24 and again at 28 days were identical. To each rat were now given 10 gm of a diet containing, instead of the rancid lard, 1 gm of the experimental fat fraction, or 1 gm of cottonseed oil containing either no vitamin A or a known amount of U.S.P. standard vitamin A acetate. To this were added, in the earlier experiments, 0.1 ml of linoleic acid containing 6.25 mg alpha-tocopherol acetate, 7.5 mg free alpha-tocopherol and 2.5 μg of vitamin D per 150 gm of diet. In the later experiments, 10 times as much of the two forms of alpha-tocopherol was given. The difference in the tocopherol supplement did not change the results.

[4] Dr. Leo Pirk of Hoffmann-La Roche, Inc., very kindly supplied most of the various vitamins for this work.

Forty-eight hours after they had been offered the supplemented diet, the animals had nearly always consumed the 10 gm offered, and they were then fed the rancid lard diet without restriction. Henceforth their weights were recorded every other day, and the maximum weight gains of the individual animals from the day previous to their being fed the supplements until they started losing weight were averaged for each group. When supplements of zero to 50 units of vitamin A acetate (U.S.P.) were fed, there was always a graded weight response. The average weight gain of the unsupplemented group was subtracted from the average weight gain of each of the groups receiving supplements, and the resulting differences were plotted on a log-log scale against the amount of supplement.

In most experiments, there was nearly a straight-line relationship between weight increase and dose, up to a supplement of 25 units. Above 25 units, the slope of the curves decreased. With a single dose of two units of vitamin A acetate, a discernible difference from results with the unsupplemented group could be produced. The probable errors obviously were greater the lower the supplement. They could have amounted to roughly $\pm$ 50% for single doses of two to 5 units and to $\pm$ 25% for supplements of from 5 to 25 units. Actually, however, when a standard curve was established for each assay of materials with unknown activities, the results agreed — perhaps somewhat fortuitously — to within $\pm$ one-half unit per gram. The results were read from the standard curve by interpolation. It must be emphasized that for each series of biological assays a new standard curve had to be prepared.

Spectroscopic determination of vitamin A

The treatment of lard and molecular distillates from lard to obtain the vitamin A factor free of other unsaponifiables which have irrelevant absorption in the spectral region 300 to 325 mμ, was similar to that described by Boldingh and Drost ('51) in their work on margarine. A quantity of sample, estimated from biological assays to contain vitamin A

activity equivalent to about 100 units, was saponified with alcoholic potash, and the unsaponifiables were extracted and chromatographed on alumina and alkaline alumina in accordance with the procedure of these workers. The size of the alumina column (but not that of the alkaline alumina column) was increased in some instances owing to comparatively large amounts of unsaponifiables involved. In other instances eluted fractions, which gave a positive Carr-Price test but which still contained interfering impurities when examined spectrophotometrically, were subjected to a second treatment on fresh alumina and alkaline alumina. Estimations of vitamin A were based only on eluates which gave a positive Carr-Price test and which also showed a maximum in spectral density at 325 mμ. The potency was calculated by multiplying $E^{1\%}_{1\,cm}$ (at 325 mμ) with the established conversion factor of 1,900.

RESULTS AND DISCUSSION

Attempts were made first to determine whether redistillation or crystallization of the original lard distillates would further concentrate the biologically active vitamin A factor.

Treatment of original distillate, O.D.-I

About 525 gm of distillate O.D.-I were subjected to molecular distillation in a cyclic still of the falling film type, about 15% being distilled and removed as 4 fractions, leaving an undistilled portion of 85%. Another portion of O.D.-I, 300 gm, was crystallized from acetone, a precipitate being removed at 0°C. and another at — 45°C., leaving some material as a filtrate fraction. The biological assays for vitamin A activity on these fractions and analyses for unsaponifiable matter are shown in table 1. Distillation was more effective than crystallization in producing a fraction with high biological assay.

These fractions were largely consumed in the biological assays; small amounts remaining were examined spectro-

photometrically without attempts to remove or separate unsaponifiables. Inflections in the spectral density curves at about 325 mμ were obtained, which were only suggestive of the possible presence of vitamin A.

Larger quantities, about 3,000 gm, of O.D.-I were similarly distilled except that about 2.5% was removed as a first fraction (B-1) and about 7.5% as a second fraction (B-2), leaving an undistilled portion (B-3) of about 90%. The biological

TABLE 1

Bioassay for vitamin A on fractions obtained from lard by molecular distillation and by crystallization

SAMPLE NO.		FRACTION	UNSAP.	BIOLOGICAL ASSAY
	Molecular distillation	%	%	*units/gm*
O.D.-I	Fr. 1 – from lard, 210°C. at 3 μ	7	4.2	4.5
A1	Fr. 1 – from O.D.-I, 85– 95°C. at 3 μ	1.5	> 35	0
A2	Fr. 2 – from O.D.-I, 95–125°C. at 3 μ	4.5	> 35	4.5
A3	Fr. 3 – from O.D.-I, 125–165°C. at 3 μ	4.0	33.2	23.0
A4	Fr. 4 – from O.D.-I, 165–200°C. at 3 μ	4.5	7.1	18.0
A5	Fr. 5 – from O.D.-I, undistilled	85	0.4	0
B1	Fr. 1 – from O.D.-I, 85–125°C. at 3 μ	2.5	38.9	2.5
B2	Fr. 2 – from O.D.-I, 125–200°C. at 3 μ	7.5	35.8	11.5
B3	Fr. 3 – from O.D.-I, undistilled	90	0.4	2.6
	Crystallization from acetone			
O.D.-I C1	Precipitate from O.D.-I, 0°C.	24	0.3	2.5
C2	Precipitate from O.D.-I, — 45°C.	61	0.6	2.6
C3	Filtrate from O.D.-I, — 45°C.	15	20.8	11.5

assays for vitamin A activity and values for unsaponifiable material are included in table 1.

Attempts to determine vitamin A in fractions B-1 and B-2 by means of chromatographic fractionation of the unsaponifiables and subsequent spectrophotometric measurements of eluted fractions resulted in only qualitative indications of its presence. Eluates were obtained which gave a weak Carr-Price test and a slight maximum at 325 mμ. The shape of the spectral curves, however, did not closely resemble that of known pure vitamin A because of too great absorption in the region 300 to 320 mμ. It was concluded that in order

to obtain more definite spectral evidence of the presence of typical vitamin A, a sample containing a lower ratio of unsaponifiables to biologically active factor would be required.

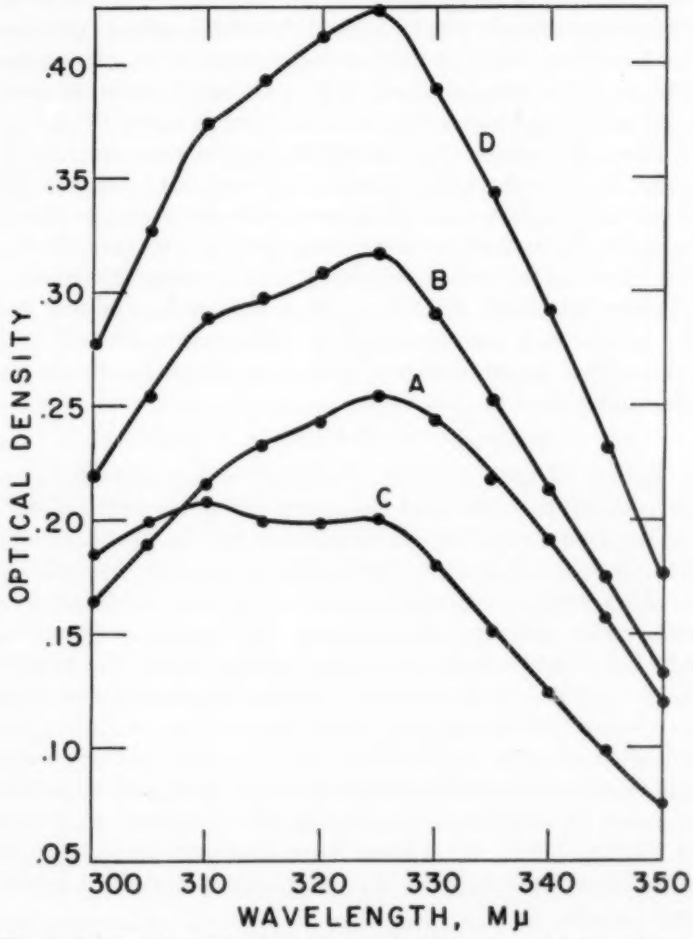

Fig. 1 Spectral absorption curves for standard vitamin A and for eluted fractions obtained by Boldingh and Drost method.

A, U.S.P. vitamin A reference standard; B, distillate from lard (O.D.-II); C, lard; D, lard to which vitamin A was added.

Fraction B-3, although relatively low in the active factor, fortunately was also low in total unsaponifiable matter. A 60-gm portion was saponified and the unsaponifiables extracted and chromatographed according to the method of Boldingh and Drost ('51). Eluted fractions giving positive Carr-Price tests were combined and examined spectrophotometrically. The curves obtained by plotting spectral density against wavelengths were almost identical to curve C (fig. 1). Assuming the absorption at 325 mμ to be due entirely to vitamin A, the calculated potency was two and eight-tenths and one and eight-tenths units per gram on separate determinations. By employing the equation of Morton and Stubbs ('48), which introduces a correction for irrelevant absorption, the values calculated were one and seven-tenths and one and seven-tenths units per gram. These values compare well with the biological assay result of two and six-tenths units per gram (table 1).

Analyses of lard distillate O.D.-II

A sample of lard distillate (O.D.-II) which had biological activity equal to 25 units of vitamin A per gram was available from previous work. It was relatively low in unsaponifiable material, containing only 2.2%. Therefore, this sample appeared to offer the greatest promise of any obtained from lard in this study for establishing the presence of typical vitamin A. The procedure of Boldingh and Drost was applied to 5-gm portions of this sample. Eluates were obtained which gave strong Carr-Price tests and a spectral curve (B) typical for known vitamin A (curve A, fig. 1). The potency calculated for duplicate determinations was 23.8 and 24.3 units per gram of distillate. Employing the equation of Morton and Stubbs ('48), the values were 23.8 and 24.0 units per gram. These values agree well with that determined by biological assay.

Analyses of lard

Biological assays have indicated that different samples of lard usually have vitamin A activity equivalent to about

four-tenths to two units per gram. An attempt was made to apply the Boldingh and Drost method for spectral determination of vitamin A to fresh lard without molecular distillation. For this purpose, 120 gm of lard were saponified, and the unsaponifiables extracted and chromatographed in accordance with the procedure. Eluates were obtained which gave positive Carr-Price tests and a spectral curve (curve C, fig. 1) resembling that of known vitamin A except for slight extraneous absorption in the region 300 to 320 mμ. The potency calculated according to the method was six-tenths units per gram (corrected value, four-tenths units per gram), a value within the range arrived at by biological assay.

It is concluded that typical vitamin A is present in lard and probably accounts for most of its biological vitamin A activity. The possibility of the presence in lard of other substances possessing similar activity but differing chemically from vitamin A is not completely excluded but is considered unlikely.

The finding of vitamin A in lard also renders unlikely previously published assertions that lard has a so-called "sparing" action on added vitamin A when it is isocalorically substituted for other nutrients, such as carbohydrate and protein. The results of this investigation may also raise a reasonable doubt about some of the reports of biological materials which are claimed to have vitamin A activity but which differ chemically from known vitamin A.

SUMMARY

Biological assays on molecular distillates from lard showed that lard contains vitamin A activity equivalent to about four-tenths to two units per gram.

Chromatographic fractionation of unsaponifiables from lard and molecular distillates from lard yielded eluates which gave positive Carr-Price tests and typical vitamin A spectral curves, except in fractions having an extremely high ratio of unsaponifiables to units of vitamin A.

It is concluded that the biological vitamin A activity of lard is largely attributable to the presence of typical vitamin A. The so-called "sparing" action of lard on utilization of added vitamin A in diets is in all probability due to the presence in lard of hitherto unrecognized typical vitamin A.

ACKNOWLEDGMENTS

We are highly indebted to Dr. Waldo C. Ault of the Eastern Regional Research Laboratory and Dr. J. J. Beaver of Columbia University for their interest and advice in connection with this work.

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NITROGEN BALANCE AND GROWTH OF THE RAT AS AFFECTED BY CERTAIN FRUITS IN THE DIET[1, 2]

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(Received for publication July 11, 1953)

The banana has long been considered a useful dietary item in both health and disease. Ever since Haas ('24) included the banana in the successful dietary management of celiac disease, this fruit has been widely used in pediatric practice (Williams and Macy, '41; Macy et al., '40; Shepherd et al., '40; Hunscher et al., '40; Kahn, '33). In an effort to gain more information regarding the nutritive value of the banana and some other fruits, the following experiments were performed with reference to N balance and growth.

METHODS

White albino rats of the Wistar strain were used. They were caged individually and where necessary the urine and feces were collected quantitatively. For dietary comparisons the adult animals were paired according to body weight (176 to 216 gm, range for whole group) and sex, and pair feeding was employed. In the growth studies weanling rats, 21 days of age, were grouped according to body weight and sex, and fed ad libitum for a period of 30 days. All animals were

[1] The data in this paper were taken from a thesis submitted by Miss Ann McLaughlin to the Graduate School of The University of Rochester in partial fulfillment of the requirements for the degree of Master of Science, 1949.

[2] This investigation was supported by a grant from Tropic Foods, Inc.

housed in a room maintained at 26 ± 0.5°C. with alternating 12-hour periods of light and darkness.

In the N balance studies the diets were fed for 7 days but urine and feces were collected only for the last 4 days in each dietary period. The lower screen and funnel of each cage were sprayed with 3% benzoic acid in 50% alcohol as a preservative for the urine. Fecal pellets were collected daily and stored in 30% H_2SO_4.

One of the fruit supplements was supplied as dried, whole, ripe banana powder.[3] For some observations this material was divided into water-soluble and water-insoluble fractions. One-pound lots of dried banana powder were extracted repeatedly with a total volume of approximately 16 l of water. The water-insoluble material was separated by filter press and Buchner funnel and finally dried in air at 75°. This material was ground in a Wiley mill until it passed a 40-mesh screen. The water-soluble material was concentrated at reduced pressure to a paste that contained approximately 42% of total solids.

In one of the growth experiments, 9% fresh fruit solids were added to 10% protein (casein) diets. These supplements were prepared by finely mincing the fresh pulp of banana, pineapple and apple.

A synthetic vitamin mixture was added to all diets in the following amounts per 100 gm of diet: thiamine · HCl, 1 mg; pyridoxine · HCl, 1 mg; riboflavin, 2 mg; niacinamide, 5 mg; calcium pantothenate, 5 mg; choline chloride, 120 mg; para-aminobenzoic acid (PABA), 10 mg; inositol, 25 mg; 2-methyl naphthoquinone, 1 mg; alpha-tocopherol, 5 mg; folic acid, 0.1 mg; biotin, 0.1 mg; vitamin A, 6,500 I.U.; vitamin D, 1,500 I.U.

RESULTS

Group 1. N balance with whole banana feeding

The composition of the diets is given in table 1. Diets Ia and Ib were protein-free. Diets IIa and IIb contained 2.5%

[3] "Meloripe" banana powder kindly supplied by Food Concentrates, Inc., New York, N. Y.

and 5.0% of protein, respectively, derived from casein. Diets IIIa and IIIb contained 2.5% and 5.0% of protein, respectively, derived in equal portions from casein and dried banana. An attempt was made to compensate for the crude fiber and

TABLE 1

Composition of diets used in N balance experiments

DIET NUMBER	I		II		III	
	a	b	a	b	a	b
Casein, gm	0	0	30	60	15	30
Sucrose, gm	773	756	757	724	510	230
Banana powder, gm	0	0	0	0	265	530
Oil,[1] gm	170	170	156	142	170	170
Cellulose flour, gm	8	16	8	16	0	0
Salts, gm	40	40	40	40	40	40
Banana ash, gm	9	18	9	18	0	0
Vitamins [2]						
Calories/kg [3]	4,660	4,595	4,615	4,503	4,615	4,503

[1] Wesson.

[2] As indicated in text, in last paragraph under "Methods."

[3] Computed.

TABLE 2

Average N balance data from adult rats fed protein-free and low-protein diets

DIET [1]	I		II		III	
	a	b	a	b	a	b
Number of rats	10	12	5	6	5	6
N intake, mg/rat/day	1.2	1.2	35.0	67.3	34.4	64.1
Fecal N, mg/rat/day	11.6	8.4	14.0	19.0	24.6	29.1
Urinary N, mg/rat/day	47.1	40.2	52.3	69.9	41.3	56.3
N balance, mg/rat/day	−57.5	−47.4	−31.6	−21.6	−31.5	−21.3

[1] See table 1 for composition of diets.

inorganic components supplied by banana in diets IIIa and IIIb by adding corresponding amounts of cellulose flour and banana ash to diets Ia, Ib, IIa and IIb. The total protein in these diets was deliberately kept low for two reasons: (1) to induce the animal to make the most of a limited protein intake, and (2) to make it possible to obtain at least one-half of the

dietary protein from dried banana, which contains approximately 5% of protein. The mean results of these feeding trials are given in table 2.

TABLE 3

Composition of diets used in testing banana fractions

DIET NUMBER	IV	V	VI	VII
Casein, gm	0	55	55	60
Fat,[1] gm	110	100	100	102
Cellulose flour, gm	40	38	5	40
Sucrose, gm	721	539	669	669
Salts, gm	40	33	40	40
Water-soluble fraction, gm	0	235	0	0
Water-insoluble fraction, gm	0	0	42	0
Water, gm	89	0	89	89
Vitamins [2]				
Cal./kg [3]	3,907	3,903	3,909	3,905

[1] Crisco.

[2] As in table 1.

[3] Computed gross energy content.

TABLE 4

Composition of banana fractions

	WATER-SOLUBLE	WATER-INSOLUBLE
	%	%
Moisture	58	0
Total solids	42	100
Protein (N × 6.25)	1.2	9.7
Ether-soluble	0.3	6.0
Cellulose	0.3	11.7
Lignin	0.3	9.7
Ash	2.1	1.5
Reducing sugar (after inversion with HCl)	23.5	0.0

Group 2. N balance with banana fraction feeding

The composition of the second group of diets is given in table 3. The data in table 4 show that the analyses do not account for all of the material present in the two banana fractions. It is evident, however, that all of the actual and

potential reducing sugar was extracted by water, and that nearly all of the cellulose and lignin remained in the water-insoluble fraction. In three of the diets used in these experiments the total protein was 5%, and in order to minimize supplementary protein effects the two banana fraction supplements were added in such amounts as to furnish less than one-tenth of the total protein. The main differences produced by the supplements were, therefore, to supply a large share of the soluble carbohydrate in diet V and a large share of the complex carbohydrate (cellulose and lignin) in diet VI. The various other components of the diet were adjusted to maintain approximate constancy of composition for diets V, VI and VII.

TABLE 5

Average N balance data from adult rats fed banana fraction supplements

DIETS	IV [1]	V	VI	VII
Number of rats	12	12	12	12
N intake, mg/rat/day	1.2	87.0	62.4	74.9
Fecal N, mg/rat/day	9.2	18.2	18.0	13.9
Urinary N, mg/rat/day	33.3	67.0	56.7	64.2
N balance, mg/rat/day	−41.3	+1.8	−12.3	−3.2

[1] For composition of diets consult table 3.

The feeding program included 7 periods of one week each arranged, for 6 rats, in the following sequence: (1) diet IV (table 3); (2) diet VII; (3) diet V; (4) diet IV; (5) diet VII; (6) diet VI; (7) diet IV.

The paired 6 rats were fed in the reverse order in periods 2 and 3 and in periods 5 and 6. The average results of the N balance study are given in table 5.

Group 3. Growth as affected by banana fractions and whole fruit supplements

One group of 8 weanling rats was fed ad libitum a complete control diet, containing 10% of casein; a second similar group was fed a similar diet which contained 323 gm of water-

soluble banana fraction per kilogram; a third group received a diet similar to the control diet but supplemented with 42 gm of water-insoluble banana fraction per kilogram. Each banana fraction furnished 0.4% of protein to the diet and this addition was compensated for by reducing the casein an equivalent amount. Differences in crude fiber content were equalized by adding or subtracting the appropriate amounts of cellulose flour. In 30 days of feeding the rats of all three groups grew at the same rate — mean initial weights were 39, 39 and 36 gm and mean final weights were 116, 126 and 112 gm for the groups on the control diet and the diets containing the water-soluble and the water-insoluble supplements, respectively.

A group of 5 weanling rats was fed ad libitum a complete diet which contained 10% of casein; three other similar groups of 5 weanlings each were fed diets which were similar except that they contained 9% of fresh fruit solids obtained from banana, pineapple and apple, respectively. All 4 groups of animals in this experiment grew at the same rate. Mean initial body weights were 41, 40, 39 and 41 gm and mean final weights, after 30 days of feeding, were 133, 134, 134 and 134 gm.

DISCUSSION

The outstanding result of this investigation is a demonstration of the high supplementary value of banana protein. The results of feeding diets IIa and IIIa (table 2) are strictly comparable and it is evident that the N balances were identical. In these experiments the diets contained 2.5% total protein and were in all respects the same except that in diet IIIa one-half of the protein was derived from dried banana. The same holds true when the total protein was doubled. Diets IIb and IIIb contained 5.0% total protein, of which one-half in diet IIIb was derived from dried banana. Here again the N balances were the same. The equality in N balance was achieved despite a significantly greater loss of fecal N ($P < 0.01$ and 0.05 for diets IIIa and IIIb, respectively) in the banana-fed rats. The urinary N was correspondingly diminished ($P = 0.07$ and 0.02 for diets IIIa and IIIb, respectively).

According to conventional computation, AN = NI — (FN —FN′), in which AN is absorbed nitrogen, NI is nitrogen intake, FN is fecal nitrogen and FN′ is fecal nitrogen on a protein-free diet. It follows from the results shown in table 2 that the utilization of absorbed N was superior in the rats fed banana as a source of one-half of their dietary N intake. A numerical expression of this superiority is obtained by plotting nitrogen balance, NB, as a function of absorbed nitrogen, AN. The slope of the line connecting the points is K, the nitrogen balance index of Allison and Anderson ('45), which may be obtained from the expression:

$$K = \frac{NB - NE_o}{AN},$$

in which NE_o is the total excretion of N on an N-free diet. The values for K on the 2.5% protein diets were 0.82 ± 0.20 and 1.78 ± 0.49 for diets IIa and IIIa, respectively. The variance was rather large and the probability that they were different is 0.10. On the 5.0% protein diets, however, $K = 0.45 \pm 0.04$ for diet IIb and 0.67 ± 0.06 for diet IIIb. All rats on the banana-supplemented diet, IIIb, yielded K values which were greater than those of their pair mates on the unsupplemented casein diet, IIb. This difference was highly significant ($P < 0.005$).

Neither the water-soluble nor the water-insoluble fractions of banana effected any detectable change in N balance (table 5). It appears, therefore, that neither the soluble carbohydrates nor the insoluble complex carbohydrates behaved any differently in these experiments than sucrose and cellulose flour, respectively.

The feeding of banana fractions as well as fresh fruit supplements of banana, pineapple and apple failed to cause an accelerated rate of growth in weanling rats.

It seems logical to conclude that the increased efficiency of utilization of absorbed N in the adult rat is associated with the supplementary value of banana protein when fed with casein at low intake. It is important to emphasize that the

total N balance was unchanged, but that the addition of banana always increased the fecal nitrogen and diminished the urinary N. The effect on fecal N is consistent with the work of Harris and Poland ('39), who reported a laxative effect of ripe fresh banana on the rat. Similar results have been reported for human subjects (Weinstein and Bogin, '39; Kantor and Cooper, '37).

Very little is known about the amino acid composition of the banana proteins. Eddy ('33) isolated enough banana protein to make some feeding tests and some observations on nitrogen distribution. In what he called the "albumin-globulin" fraction the following amino acids were present: arginine (11.08%), histidine (0.39%) and lysine (3.26%). It would be most interesting to know the concentration of the other essential amino acids, especially methionine because it is a very effective supplement for casein (Brush et al., '47).

SUMMARY

Nitrogen balance observations were made on adult rats which were pair-fed diets low in protein (2.5% and 5.0%). Casein was compared with an isonitrogenous mixture of casein (50%) and banana protein (50%). The nitrogen balance was unaffected by adding the fruit protein. The fecal N, however, was greatly increased and the urinary N correspondingly decreased when casein and banana protein were fed together. The nitrogen balance index of absorbed nitrogen was, therefore, increased as the result of supplementing casein with banana protein.

Feeding water-soluble as well as water-insoluble fractions of dried banana as supplements to a diet containing 10% of protein from casein failed to affect the N balance of adult rats.

The addition of 9% fresh fruit pulp solids from banana, pineapple and apple failed to affect significantly the growth of weanling rats fed ad libitum.

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THE RETENTION OF ESSENTIAL AMINO ACIDS BY THE GROWING CHICK[1, 2, 3]

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(Received for publication July 17, 1953)

Although information is available concerning the requirements of the growing chick for the essential amino acids, very little is known as to what quantities of these amino acids consumed in a ration are retained in the body of the chick. Grau ('47) has reported the retention of DL-phenylalanine by the 4-week-old chick to be 35%. The object of the present investigation was to determine what percentages of the essential amino acids consumed are retained by the chick when fed rations of known amino acid content, and to gain further information on the requirements of the chick for the indispensable amino acids.

PROCEDURE

The two rations contained natural protein sources only. In the first experiment, ration A, a practical type of chick starter ration, having the following percentage composition, was used: yellow corn meal, 44.28; soybean oil meal, 20; wheat

[1] Journal Series paper of the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, New Brunswick.

[2] This study was made possible by a grant from the Cooperative G.L.F. Exchange, Ithaca, New York.

[3] Presented before the American Institute of Nutrition, New York, N. Y., April 14–18, 1952.

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standard middlings, 10; fine ground oats, 5; dried corn distillers' solubles, 4; dried whey, 4; fish meal, 4; meat scrap, 3; dehydrated alfalfa meal, 3; dicalcium phosphate, 1; ground limestone, 1; riboflavin supplement, 0.4; iodized salt, 0.25; manganese sulfate, 0.019; and vitamin D-activated animal sterol, 0.05. The ration used in the second experiment, ration B, had the following percentage composition: ground whole oats, 24; ground whole corn, 21.33; ground whole wheat, 16; dried lean beef, 4; dried whole egg, 4; dried beef liver, 4; fish meal, 4; calcium carbonate, 1.6; vitamin mixture (Scott et al., '47), 0.81; mineral mixture (Briggs, '46), 0.4; manganese sulfate, 0.02; and cornstarch to make up to 100.

In both experiments, White Leghorn chicks were reared to one week of age in a brooder on a chick starter ration (ration A). A representative group of chicks was then killed by gassing, the contents of the digestive tracts removed, and the carcasses weighed and frozen. Another group was placed in heated, individual cages equipped with feed cups designed for the quantitative determination of feed consumption.

In the experiment using ration A, both groups of chicks consisted of 5 pullets and 5 cockerels, whereas in the experiment using ration B, 5 cockerels were employed in each group. Feed consumption was recorded during both experiments. With the first quantity of ration fed a carmine marker was included, and excreta collection was begun when the marker appeared in the feces. The excreta, collected on glass plates under the cages, were removed daily and dried at 70°C. When each chick had gained 160 to 170 gm (25 to 30 gm of protein), another carmine marker was fed, and upon appearance of the marker in the feces, excreta collection was stopped. The chick was then killed by being gassed, the contents of the digestive tract removed, and the chick carcass weighed and frozen.

Each frozen carcass was chopped into small pieces on a glass plate, dried at 70°C. for 15 to 20 hours, ground in a meat grinder and extracted with ethyl ether. The extracted carcasses and the dried excreta of each chick were then in-

dividually ground to a fine powder in a ball-mill prior to hydrolysis for the amino acid assays. The chick rations were also ball-milled before being assayed.

Samples of the powdered chick carcasses, chick excreta and rations were hydrolyzed and assayed for the essential amino acids by the method of Stokes et al. ('45), except that dry amino acid mixtures (Rockland and Dunn, '46) and a dry vitamin-glucose mixture were used instead of solutions. Also, the time of autoclaving the assay tubes was reduced from 13 minutes at 15 lb. of pressure to 3½ minutes at 16 to 17 lb., with a period of pressure reduction of 8 to 10 minutes, as recommended by Toennies and Gallant ('48), to minimize possible inactivation of the amino acids by the so-called browning reaction.

The total nitrogen of the carcass and excreta of each chick and of the chick rations was determined by the macro-Kjeldahl procedure (Association of Official Agricultural Chemists, '45), using mercury as the catalyst during digestion, which was continued for three hours after clearing.

RESULTS AND DISCUSSION

In table 1 are presented the essential amino acid and crude protein contents of the rations. The crude protein content of ration B was 25% lower than that of ration A and 20% lower than the recommended level for optimum growth, which is usually considered to be 20%. The percentages of the essential amino acids in ration A were equal to or above the recommended allowances in every case except for methionine and tryptophan, which were 22% and 4% lower, respectively. In ration B, however, there were deficiencies of 9 to 32% in the arginine, leucine, methionine, phenylalanine and tryptophan contents in comparison with the recommended levels.

The similarity in the essential amino acid contents of the two rations when calculated on the basis of crude protein should be noted. It is of interest that ration A, containing chiefly plant protein sources, and ration B, containing rela-

TABLE 1

Essential amino acid and crude protein contents of chick rations

AMINO ACID	RECOMMENDED ALLOWANCE [1]	RATION A (CHICK STARTER)			RATION B (HIGH ANIMAL PROTEIN)		
		Amino acids [2]	Difference from recommended allowance	Amino acids calculated to 16% N	Amino acids [2]	Difference from recommended allowance	Amino acids calculated to 16% N
	%	%	%	%	%	%	%
Leucine	1.4	1.77	+ 26	8.1	1.28	— 9	7.8
Arginine	1.2	1.26	+ 5	5.7	0.82	— 32	5.0
Lysine	0.9	1.07	+ 19	4.9	0.90	0	5.5
Phenylalanine	0.9 (+ 0.7 tyrosine)	0.95	+ 5	4.3	0.74	— 18	4.5
Valine	0.8	1.03	+ 29	4.7	0.82	+ 3	5.0
Isoleucine	0.6	0.91	+ 52	4.2	0.73	+ 21	4.5
Threonine	0.6	0.81	+ 35	3.7	0.64	+ 7	3.9
Methionine	0.5 (+ 0.4 cystine)	0.39	— 22	1.78	0.35	— 30	2.14
Histidine	0.3	0.40	+ 33	1.82	0.31	+ 3	1.9
Tryptophan	0.25	0.24	— 4	1.09	0.19	— 24	1.17
Crude protein (N × 6.25)		21.9			16.3		

[1] National Research Council ('50).

[2] In ration as fed, average of two determinations.

tively large quantities of animal proteins, should have almost the same pattern of essential amino acids.

The data in tables 1 and 2 reveal that the pattern of essential amino acids in the crude protein of the carcasses of each of the groups of chicks showed considerable similarity to the pattern of the acids in the crude protein of the rations consumed. Present-day feeds, such as the chick starter ration,

TABLE 2

Essential amino acid and nitrogen contents of the ether-extracted chick carcasses[1] and the excreta

AMINO ACID	RATION A			RATION B		
	Week-old chick [2]	4- to 5-week-old chick [2]	Chick excreta [2]	Week-old chick [3]	4-week-old chick [3]	Chick excreta [3]
	%	%	%	%	%	%
Leucine	7.3	7.1	0.87	6.9	6.9	0.59
Arginine	6.6	6.7	0.44	6.7	6.4	1.35
Lysine	6.8	6.4	0.68	6.1	6.0	0.64
Valine	4.8	5.2	0.57	5.0	5.0	0.46
Threonine	4.4	4.4	0.50	4.5	4.6	0.46
Phenylalanine	4.4	4.1	0.39	4.2	4.2	0.40
Isoleucine	4.2	4.5	0.49	4.1	4.1	0.37
Methionine	2.08	1.88	0.15	1.94	1.90	0.15
Histidine	1.71	1.88	0.18	1.82	1.79	0.16
Tryptophan	1.03	0.93	0.11	0.94	0.85	0.09
Total amino acids	43.32	43.09		42.20	41.74	
Amino acid N, calculated		6.95			6.68	
Per cent of total N		58.40			54.84	
Total nitrogen	11.14	11.90	6.10	12.00	12.18	4.55

[1] Amino acid content calculated to 16% N.

[2] Average of 10 chicks.

[3] Average of 5 chicks.

have been developed to a fairly high degree of efficiency, and it is quite possible that this efficiency depends in part upon a similar pattern of amino acids in the feed and in the carcass. The similarity, however, must be considered accidental, since the feeds were not designed to provide a protein whose essential amino acid content resembled closely that of the chick's body. The fact that many proteins have essential amino acid contents ranging from a high value for leucine to a low one for tryptophan (Block and Bolling, '51) quite probably caused this similarity in the patterns of amino acids.

Furthermore (table 2), for the essential amino acid contents of the crude protein of the carcasses of the chicks on each ration there was close agreement, any differences between groups being within the range of experimental error. The values for the one-week-old chicks were nearly identical with those found for chicks 4 to 5 weeks of age. These results are of the same order as the calculated values for two whole mature chickens reported by Dunn et al. ('49), although they are generally slightly higher. In the 4- to 5-week-old chicks on ration A the calculated nitrogen content of the essential amino acid of the carcass was 6.95%, and this value is 58.40% of the total nitrogen of the carcass. For ration B, the values are of the same order, being 6.68% and 54.84%, respectively. These findings confirm the observations of others on the constancy of the amino acid composition of proteins. Grau ('47), after feeding diets with wide ranges of phenylalanine and tyrosine, found no change in the percentage of either amino acid in the chick carcass.

The total nitrogen contents of the carcasses shown in table 2 indicate that the crude protein of the 4-week-old chicks was essentially the same for both groups.

For the excreta of the chicks fed ration B, the total nitrogen content of 4.55% was considerably less than the value of 6.10% for ration A. The decrease in the excretion of essential amino acids (table 3) on ration B, as well as the excretion of less total nitrogen, reflects the lower nitrogen content of ration B and its more efficient utilization.

From the data in tables 1 and 2 the percentage of each essential amino acid consumed which was retained, excreted or catabolized was calculated and the results are presented in table 3. The quantity of an amino acid gained by the carcass

TABLE 3

The retention and excretion of the essential amino acids and nitrogen by the growing chick

AMINO ACID	RATION A [1]				RATION B [2]			
	Retention		Excretion	Unaccounted for (catabolized)	Retention		Excretion	Unaccounted for (catabolized)
	%	C.V.% [3]	%	%	%	C.V.% [3]	%	%
Lysine	43	8.8	21	36	61	6.4	15	24
Threonine	39	7.7	20	41	63	1.6	15	22
Arginine	38	6.2	11	51	71	6.5	9	20
Valine	38	6.6	19	43	55	2.4	12	33
Isoleucine	37	6.7	18	45	50	3.2	11	39
Histidine	35	7.8	15	50	52	3.7	11	37
Methionine	34	9.4	12	54	49	3.0	9	42
Phenylalanine	30	6.1	14	56	50	4.7	11	39
Leucine	29	6.9	16	55	49	3.2	10	41
Tryptophan	27	8.7	15	58	39	11.0	10	51
Nitrogen	33	6.1	60	7	56	2.5	38	6

[1] Average of 9 chicks.

[2] Average of 5 chicks.

[3] Coefficient of variation $= \frac{\sqrt{\frac{\Sigma d^2}{(n-1)}}}{\text{Mean}} \times 100.$

is the difference between that found in the chicks sacrificed at the beginning of the feeding trial and that of the 4- to 5-week-old birds. The percentage catabolized was not determined by analysis but was obtained by subtracting the sum of the percentages retained and excreted from 100.

On ration A the chick retained in its body from 27 to 43% of the essential amino acids consumed, excreted 11 to 21%,

and catabolized 36 to 58%. For ration B the corresponding values were 39 to 71% retained, 9 to 15% excreted, and 20 to 51% catabolized. In both experiments the percentage unaccounted for, and presumably catabolized, generally varied inversely with the percentage retained. For ration A the percentage excreted tended to decrease as the percentage retention became lower, whereas for ration B the excretion was more nearly constant.

The percentages of essential amino acids retained by the chicks fed ration B were considerably higher than those for the birds fed ration A. Some of the increase in percentage retention was due to the lower levels of the amino acids in ration B and the smaller quantity of this ration consumed per gram of body protein gained. For the amino acids present in the rations in quantities less than the recommended allowances, a higher percentage retention would be expected. That this was not the case is shown by the fact (tables 1 and 3) that on ration A the methionine content was 22% less than the recommended allowance, yet for 6 amino acids, present in adequate amounts, the retention values were higher. Furthermore, for ration B, 4 of the 5 amino acids present in the ration in less than the recommended quantities showed retention values lower than those provided in adequate amounts.

Tryptophan was retained in the lowest percentage on each ration, and the retention values for the other acids ranged above that of tryptophan in essentially the same sequence in both groups. In general, the percentages of amino acids unaccounted for (catabolized) varied inversely with the percentage retention, whereas the percentages excreted tended more toward a constant level. Since there was such a variation in the percentage catabolized, it would appear that the chick shows considerable variation in the efficiency with which it uses the different essential amino acids for tissue formation. Furthermore, the rather high percentages catabolized, ranging from 36 to 58% on ration A and 20 to 51% on ration B, indicate either that the over-all process of converting dietary to body protein is fairly inefficient, or that the recom-

mended allowances for many of the amino acids have been set too high. Since the recommendations have been studied rather carefully, the latter possibility seems unlikely.

Coefficients of variation, with one exception, of less than 10% (table 3) for the retention values show good agreement among the results obtained for individual birds.

SUMMARY

The percentage retentions of essential amino acids were determined for the growing chick when fed a chick starter ration (ration A) whose protein sources were largely of plant origin, and ration B, containing high percentages of animal proteins but partially deficient in certain essential amino acids.

On ration A, 24 to 43% of the essential amino acids consumed was retained by the body, 11 to 21% was excreted, and 36 to 58% was unaccounted for and considered to have been catabolized. Corresponding values for ration B were 39 to 71%, 9 to 15%, and 20 to 51%. Of the total nitrogen consumed, 56% was retained, 38% excreted and 6% unaccounted for.

There was a marked similarity in the patterns of the essential amino acids in the total protein of the chick carcasses and the rations. Also, the patterns for the two groups of chicks were essentially the same.

ACKNOWLEDGMENT

We wish to thank Henningsen Bros., Inc., New York, N. Y., for a supply of dried whole egg.

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INTESTINAL SYNTHESIS OF BIOTIN IN THE RAT[1]

EFFECT OF DEFICIENCIES OF CERTAIN B VITAMINS AND OF SULFASUXADINE AND TERRAMYCIN

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(Received for publication June 22, 1953)

The nutritional aspects of the intestinal microflora must include the nutrition of the organisms per se. It is known that diet influences the number and type of bacteria present in the intestinal tract of animals. Porter and Rettger ('40) found that the intestinal flora in rats on high protein diets was predominately protoeolytic in nature, whereas a largely carbohydrate diet influenced a change toward fermentative bacteria.

Guerrant et al. ('34, '35) have reported lower requirements of thiamine and riboflavin when dextrinized cornstarch was used as the sole carbohydrate component in a purified diet. They suggested that this decreased requirement might be due to increased intestinal synthesis of these vitamins.

Skeggs and Wright ('46) and Couch et al. ('48) found a substantial change in biotin excretion, depending upon the carbohydrate used. Dextrin was found to promote a higher rate of synthesis than sucrose, glucose, cerelose or cornstarch. Gall et al. ('48) also found a marked change in the intestinal flora, both in type and number, when the carbohydrate component was changed.

[1] This investigation was supported in part by funds provided for biological and medical research by the State of Washington Initiative Measure 171.

Scientific Paper 1235, Washington Agricultural Experiment Stations, Pullman. Project 1041.

The addition or deletion of one vitamin can change the rate of synthesis of other vitamins. This was shown by Wegner et al. ('40). They reported that the addition of thiamine to a calf ration apparently increased the synthesis of riboflavin, pantothenic acid, pyridoxine and biotin. Neilson and Elvehjem ('42) found higher biotin synthesis in rats on stock diets than in those on a diet deficient in pantothenic acid or riboflavin. Woolley ('42) reported a greater synthesis of biotin in mice when pantothenic acid was added to the diet. He also found that inositol-deficient mice were benefitted by being fed pantothenic acid, thus indicating that the synthesis of inositol was enhanced.

Cunha et al. ('46) found that the addition of inositol and sulfathaladine to a basal purified ration for pigs alleviated, to a large extent, the deficiency symptoms which were prevented by the administration of biotin. Cunha et al. ('48) likewise reported that biotin and folic acid in combination were more effective than folic acid alone in counteracting the effect of a crude folic acid antagonist.

The biotin deficiency syndrome in rats was also demonstrated by Emerson and Keresztesy ('46) when 0.75% Marfanil (4-homosulfanilamide hydrochloride) was fed with a purified diet. They also reported an initial lowering in both the coliform and total bacterial count in the intestinal tract which returned to normal after 10 to 12 days of trial.

The use of bacteriostatic agents has been a convenient tool in the study of intestinal synthesis of the B vitamins as well as of their interrelationship. The incorporation of these drugs into the diet and the maintenance in the diet of all the factors except those under investigation make a direct balance method possible.

That the antibiotics are bacteriostatic agents and act in nutrition through this method has been shown by Reyniers et al. ('52) by feeding antibiotics to germ-free chicks and turkeys and not finding any beneficial growth response such as is found in normal animals having an established intestinal flora.

Emerson and Smith ('45) have reported biotin deficiency in rats when streptomycin was added to a purified ration. Feeding this antibiotic also produced a decrease in the total bacterial count in the intestinal tract which was of longer duration than was brought about by the administration of sulfasuxadine. A lowering of the amount of biotin synthesized by the intestinal flora was observed by them at the same time.

EXPERIMENTAL

The purified diets used were those employed by Heinemann et al. ('46). The rations were shown to sustain a good rate of growth in pigs when supplemented by B complex vitamins.

The basal diet consisted of, in per cent: vitamin-test casein [2] 26.11; sucrose 57.7; lard 11.0; and mineral mix (Osborn and Mendel; Wesson's modification) 5.2. The control ration was supplemented by the following vitamins, in milligrams per 100 gm of diet: thiamine hydrochloride, 0.3; riboflavin 0.3; niacin 2.5; Ca pantothenate 1.0; pyridoxine 0.3; choline 300.0; and menadione 0.1.

All rats received 500 U.S.P. units of vitamin A and 500 U.S.P. units of vitamin D each week. All rations were refrigerated.

Weanling female rats were used. A 25-day pre-trial conditioning period was allowed before the animals were placed in metabolism cages for the 12- to 14-day balance trials. Urine and feces were collected under 3% acetic acid, and homogenized aliquots were taken of the 12- to 14-day composite.

Two sets of trials were conducted. The rats in the first two trials were given a stock diet and were managed as previously described. This experiment was undertaken to study: (1) interrelationship of biotin and folic acid, and (2) the effect of sulfasuxadine and terramycin separately and in combination upon the intestinal synthesis of biotin. The microbiological assay for biotin of Wright and Skeggs ('44) as modified by these authors (Skeggs and Wright, '46) and using *L. arabinosus* was used.

[2] General Biochemicals, Inc.

In trial 2 (two separate trials) the basal diet was changed so that its composition was, in per cent: vitamin-free casein [3] 26; sucrose 10; starch (raw potato) 54; corn oil 5; minerals (Wesson's modification) 5; plus all the known vitamins except biotin. Added to this ration with different groups of rats were: (1) sulfasuxadine, 2%; (2) terramycin (15 mg/kg basal); and (3) 2% sulfasuxadine plus 15 mg terramycin per kilogram of feed.

In the replicate of trial 2 (designated as 2B) the starch content of the diet was reduced to 35% and the sucrose increased to 24%; otherwise the trial conditions remained the same.

RESULTS AND DISCUSSION

The data from the biotin balance trials are given in tables 1, 2 and 3. It can be seen that synthesis of this vitamin occurred in the intestinal tract of rats receiving the purified basal diet but without biotin. When sulfasuxadine was added at a 2% level, the total synthesis, as well as the quantity of the vitamin excreted per day, was diminished to about 50% that of the group not receiving the drug (based on dry weight of excreta). Synthesis of the vitamin was not completely inhibited even when the sulfa drug was included in the diet.

The addition of folic acid in the presence of sulfasuxadine produced no increase in the amount of non-dietary biotin excreted.

Thiamine deficiency produced a decrease in the total amount of excreted, non-dietary biotin but not in the quantity produced per gram of excreta eliminated, when calculated on the same basis for the control lot. This finding was interpreted as an indication that the lowering of biotin synthesis in this case was brought about because of the anorexia produced by the thiamine deficiency. Lowered food intake resulted in the excretion of a much smaller average amount of elimination products (60%). Because of the fact that there was no significant difference in the amount of biotin excreted

[3] See footnote 2, page 425.

TABLE 1

Biotin balance trials

Interrelationship with folic acid in the presence of sulfasuxadine

TREATMENT	RAT NO.	NET BIOTIN EXCRETED	EXTRA-DIETARY BIOTIN/DAY [1]	VALUE COMPARED WITH CONTROLS	BIOTIN EXCRETED PER RAT PER GRAM OF EXCRETA	VALUE COMPARED WITH CONTROLS
		μg	μg		μg	
Purified basal	2	9.13	0.76		0.71	
control	3	10.42	0.86		0.92	
	5	7.48	0.63		0.71	
	10	7.97	0.67		0.76	
Purified basal	4	3.10	0.26		0.30	
+ 2% sulfasuxadine	9	3.53	0.30	$t = 6.23$ [2]	0.34	$t = 6.43$ [2]
	16	4.57	0.38		0.46	
	6	4.67	0.39		0.47	
Purified basal	1	3.17	0.26		0.33	
+ 2% sulfasuxadine	7	2.95	0.28	$t = 9.39$ [2]	0.35	$t = 9.81$ [2]
+ folic acid, 2 μg/day	13	2.93	0.24		0.32	
	23	3.05	0.26		0.34	

[1] Twelve-day trials. Difference between second and third groups $t_0 = 1.64$ (not significant).

[2] $P < 0.05$.

TABLE 2

Biotin balance trials

Effect of deficiency of certain of the B complex vitamins

TREATMENT	RAT NO.	NET BIOTIN EXCRETED	EXTRA-DIETARY BIOTIN/DAY [1]	VALUE COMPARED WITH CONTROLS	BIOTIN EXCRETED PER GRAM OF EXCRETA	
		μg	μg		μg	
Purified basal	1	6.44	0.64		0.92	
6 B vitamins	2	6.44	0.64		0.92	
(control)	3	7.36	0.73		1.34	
	4	7.91	0.79		1.25	
Purified basal	5	3.44	0.34		0.82	
5 B vitamins,	6	3.48	0.35 [2]		0.83	$t = 0.32$
no thiamine	7	4.28	0.43	$t = 7.87$ [3]	1.77	
	8	3.42	0.34 [2]		1.15	
Purified basal	9	2.33	0.23		0.47	
5 B vitamins,	10	2.03	0.20		0.40	$t = 5.71$ [3]
no riboflavin	11	3.35	0.34	$t = 7.95$ [3]	0.70	
	12	3.36	0.34		0.70	
Purified basal	13	4.38	0.44		0.59	
5 B vitamins,	14	4.62	0.46		0.62	$t = 6.84$ [3]
no niacin	15	3.31	0.33	$t = 6.12$ [3]	0.57	
	16	3.37	0.34		0.58	
Purified basal	17	3.62	0.36		0.63	
5 B vitamins,	18	3.68	0.37		0.63	$t = 7.54$ [3]
no pantothenic acid	19	2.71	0.27	$t = 8.22$ [3]	0.46	
	20	2.61	0.26		0.46	
Purified basal	21	5.35	0.54		0.92	
5 B vitamins,	22	5.07	0.51		0.88	$t = 2.56$
no pyridoxine	23	4.53	0.45	$t = 4.70$ [3]	1.00	
	24	4.98	0.50		1.08	

[1] Twelve-day trials.
[2] Ten-day trials.
[3] $P < 0.05$.

TABLE 3

Biotin balance trials

Effect of sulfasuxadine and terramycin on synthesis of biotin

TREATMENT	RAT NO.	TOTAL BIOTIN EXCRETED	EXTRA-DIETARY BIOTIN PER DAY [1]		BIOTIN EXCRETED PER RAT PER GRAM OF EXCRETA	
		μg	μg		μg	
Purified basal	1	88.57	7.24		1.78	
	2	87.35	7.28		1.54	
	3	77.30	6.44		1.81	
Purified basal	4	32.80	2.73		0.93	
2% sulfasuxadine	5	35.43	2.95	$t = 14.81$ [2]	1.28	$t = 3.19$ [3]
	6	33.74	2.81		0.78	
Purified basal — terramycin	7	38.07	3.17		0.78	
(15 mg/kg ration)	8	28.07	2.34	$t = 11.35$ [2]	0.54	$t = 6.06$ [3]
	9	28.83	2.40		0.56	
Purified basal	10	13.75	1.15		0.27	
2% sulfasuxadine —	11	12.67	1.06	$t = 10.70$ [2]	0.27	$t = 8.89$ [3]
terramycin (15 mg/kg ration)	12	12.01	1.00		0.24	

[1] Twelve-day trials.

[2] $P < 0.01$.

[3] $P < 0.05$.

per gram of excreta between the control lot and the thiamine-deficient lot, the total synthesis of the vitamin must have been limited by the indirect cause, anorexia, instead of by reduction of any of the predominant synthesizing organisms.

Deficiencies of riboflavin, niacin and pantothenic acid caused a lowering of both the total excretion of non-dietary biotin and of the quantity excreted per gram of dry excreta, indicating that the absence of these vitamins from the diet exerted a direct effect upon the synthesizing ability of the intestinal bacteria.

The absence of pyridoxine in the diet resulted in no change in the intestinal synthesis of biotin, either in terms of total excretion or on the gram basis of excreta.

The fact that the amount or bulk of fecal material present in the intestine may possibly limit the bacterial growth, and therefore synthesis, would seem to be in agreement with the theory of Guerrant et al. ('35) that the increase of synthesis which was observed with the use of dextrin was due to the production of a bulkier feces. It might likewise explain, at least partially, the increased synthesis reported to have been found with the use of natural diets containing bulk in the form of indigestible cellulose, and so forth, when compared to purified rations containing very little indigestible fiber.

In cases where synthesis of biotin was lowered both in total amount and also in the quantity per gram of excreta, it can be concluded that the effect upon synthesis of the vitamin must have been caused by failure to satisfy the nutritive requirements of the predominating synthesizing organism. Synthesis, however, was not altogether stopped under any of the conditions of the experiments, probably because of the fact that total synthesis is the summation of the production of several or many types of organisms having different nutritive requirements and even different susceptibility to the bacteriostatic action of sulfasuxadine. Obviously, the total synthesis of the vitamin is of primary significance as far as the general nutritional status of the animal is concerned. Whether the amount of synthesized vitamin is lowered because of

general lowering of the numbers of synthesizing organisms present, or due to the inhibition of the growth of specific synthesizing bacteria, the resulting effect upon the animal is the same.

The intestinal synthesis of biotin was increased in the basal group when part of the sucrose was replaced by raw potato starch, as is shown by table 3. These data confirm the work of Couch et al. ('48) and other authors previously discussed who noted variation of intestinal synthesis depending on the type of carbohydrate used. Synthesis was increased when starch was used, even in the presence of sulfasuxadine.

The bacteriostatic action of the terramycin was similar in direction and degree to that of the sulfasuxadine in inhibiting the synthesis of biotin.

A combination of terramycin and sulfasuxadine almost completely inhibited intestinal biotin synthesis. These results are similar to those reported by Monson et al. ('52), although they are not directly comparable because these workers used rations adequate in the vitamins being tested.

SUMMARY AND CONCLUSIONS

1. The microbiological synthesis of biotin in the intestinal tract of the rat, as measured by direct balance studies, was inhibited about one-half when succinylsulfathiozole was added to a purified diet at a 2% level.

2. Biotin synthesis was found to be diminished when the diet was free of riboflavin, niacin and pantothenic acid. The excretion of non-dietary biotin was lower both in total amount and when calculated in micrograms per gram (dry weight) of excreta eliminated during the trials.

3. The total amount of biotin synthesized by the intestinal flora of rats suffering a thiamine deficiency was diminished. The amount excreted per gram of excreta, however, was comparable to that of the control lot. It was concluded that, in this case, the lowering of the excretion of non-dietary biotin was caused by the anorexia of the thiamine deficiency and the resulting smaller amount of material excreted, and was

not a direct effect of the vitamin deficiency upon the synthesizing microflora.

4. A pyridoxine deficiency caused no significant change in either the total biotin excreted or in the quantity excreted per gram of excreta during the trial.

5. Terramycin added to the biotin-free diet diminished the synthesis of biotin to an extent similar to 2% sulfasuxadine; and the combination of both terramycin and sulfasuxadine lowered the synthesis to about 1 μg/day, indicating separate inhibiting action.

6. When starch replaced sucrose, there was an increased synthesis of biotin in the intestinal tract.

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THE CONCEPT OF AN "APPETITE QUOTIENT" FOR THE INTERPRETATION OF AD LIBITUM FEEDING EXPERIMENTS

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(Received for publication July 30, 1953)

It is a commonplace of "ad libitum" feeding experiments that the animals receiving one diet will eat more food and grow more rapidly than those receiving another diet. The question in dispute has then been whether the animals that grew faster did so because they ate more (Graves, '45; Mitchell, '27) or whether they ate more because they were growing more (Chick, '47).

The first method presented as a solution to this problem was that of pair feeding. This method, although it does eliminate differences due to variations in the appetite of animals receiving different treatments, has been rightly criticized on the grounds of reducing the effects of real differences between diets, as the rapidly-growing animal will have to use more of its ration for maintenance than will the lighter, pair-fed animal on the more deficient diet. A further refinement suggested for use with laboratory animals has been the adoption of feeding-scales based on the weight of the animal at any time (Carpenter and Porter, '52; Sherwood and Weldon, '53).

However, it is generally agreed that all these methods are more laborious than ad libitum feeding, and it seems likely that most work will continue to be carried out by this method. Certainly experiments carried out in this way in the past have to be used as sources of information. This is particularly true of work aimed at the study of vitamin-deficient diets.

The most usual method of attempting to eliminate the "food intake" factor in these experiments has been to calculate "food conversion efficiencies" (grams of weight gain/grams of feed eaten) for each diet. If this measure differs significantly for different dietary treatments, then it is often assumed that the possible criticism of the results as being explained by "palatability" or "appetite" differences has been averted.

Further consideration shows this argument to be invalid. As has been stated by many authors, a reduction of food intake will decrease the proportion of the food available for growth, and regardless of the exact relationship between the efficiency of utilization of this fraction and the plane of nutrition, it is obvious that as the feed intake is reduced to the level required for maintenance alone, the "food conversion efficiency" will necessarily approach zero.

On the other hand, criticism of an ad libitum feeding trial might be averted if it could be demonstrated that the diets under comparison were in fact equally palatable for the experimental animals, and that they ate them in quantities proportional to their maintenance requirements over the experimental period. From the study of their own results and of those reported by other workers, Hegsted and Haffenreffer ('49) conclude that the feed consumption of a growing rat will often satisfy these criteria — at any rate for series of diets differing only in their protein content.

These workers found that the nutrient intake (measured as metabolizable energy) of growing rats varied as their mean body weight raised to the 0.88 power; and the results of Brody ('45) also indicate that the basal metabolic rate of comparable rats varies with their body weight raised to a power not significantly different from 0.88.

It is the intention of the present paper only to suggest a terminology and a standard method of mathematical treatment to test whether or not rats in an ad libitum feeding trial have "had the same level of food intake" — a condition defined by Kleiber ('47) as being such that "the rate of intake of metabolizable energy is the same multiple of the standard metabolic

rate," and here as being such that "the rate of intake of metabolizable energy is the same proportion to (body weight)$^{0.88}$."

I have given this multiple the name "appetite quotient," and use the notation Q_x as the "appetite quotient" for diet$_x$. At any time in the experiment, theoretically,

$$\underset{\text{[rate of food consumption]}}{\frac{\Delta F}{\Delta t}} \cdot \underset{\text{[metabolizable Calories per gram of feed]}}{C_x} = \underset{\text{[appetite quotient]}}{Q_x} \cdot \underset{\text{[body weight]}}{W^{0.88}}$$

In fact, food consumption is irregular from hour to hour, or even day to day, so that the calculation is most accurately made over the whole period of the experiment (T), measured in days.

$$\text{(Total feed eaten) } F \times C_x = Q_x \int_0^T W^{0.88} dt,$$

$$\text{or, } Q_x = \frac{F \times C_x}{\int_0^T W^{0.88} dt}.$$

The term $W^{0.88}dt$ is, of course, the area under the graph of $W^{0.88}$ plotted against time. For experiments of two to 5 weeks in duration this graph is sufficiently straight for a reasonable approximation to be obtained with twice-weekly weighings.

The value obtained for Q_x refers to an individual animal; the mean for all the animals must be obtained from a final average of the Q_x calculated for each individual, if the standard error of the estimate is to be calculated.

In many vitamin experiments the metabolizable energy of each ration will be the same, although its absolute value is unknown. There is no need for this value, therefore, in order to calculate whether or not the "appetite quotients" differ significantly from one treatment to another.

Again, in many vitamin experiments a severe deficiency may terminate with a period of refusal to eat, and death. Under such conditions the "appetite quotient" will be sub-normal if the whole period be taken. However, it is possible to test whether the failure of the ration is primarily due to loss of

appetite by analyzing only up to the point where a growth depression first reaches statistical significance.

EXAMPLE

This method of analysis has been applied to an experiment for which the growth rates have already been published (Laguna and Carpenter, '51; experiment 1). As is shown in figure 1 of that paper and in table 1 of the present paper, the results indicated that lime-treated corn allows a growth rate only obtained with a similar raw-corn ration when the latter is

TABLE 1

Growth and feed-consumption of rats, according to diet, in 29 days, in experiment 1. (Laguna and Carpenter, '51; each figure is the mean for 8 individually caged animals)

DIET NO.	TYPE OF CORN	NIACIN	INITIAL WT.	FINAL WT.	WT. GAIN	MEAN FEED EATEN/DAY	"FOOD CONVERSION EFFICIENCY" — WT. GAIN/GM FEED EATEN
		mg %	*gm*	*gm*	*gm*	*gm*	
99	Untreated	..	47.6	82.7	35.1	8.1	0.145
101	Lime-cooked	..	46.1	125.6	79.5	10.0	0.236
100	Untreated	3	46.6	117.1	70.5	9.6	0.244
102	Lime-cooked	3	47.0	122.7	75.7	9.6	0.263

supplemented with niacin. Cravioto, Massieu, Cravioto and Figueroa ('52) have pointed out that these effects, and also those in the original work of Krehl, Teply and Elvehjem ('45), demonstrating a higher growth rate with niacin supplementation of high-corn rations, can possibly be explained by differences in feed consumption, unless there is evidence to the contrary.

As is shown in table 1, the rats on the treatment where growth was slow also ate less; and reasons have already been given against the use of "food conversion efficiencies" to eliminate this effect.

In table 2 the means are set out for calculation of the "appetite quotients" on the different treatments. The calculations have been repeated with each individual to allow of a statistical analysis, and it is seen that there was in fact no significant difference among the "appetite quotients" of the rats receiving the 4 rations, and that the mean for the "deficient" ration (No. 99) is near the center of the range. If the argument set forth above be accepted, then it may be concluded that the observed differences in growth are not to be explained by differ-

TABLE 2

Calculation of "appetite quotients" for experiment 1 (Laguna and Carpenter, '51)

DIET NO.	MEAN FEED EATEN PER HEAD	MEAN $\int_0^{29} W^{0.88}dt$	Q/C [1] (i.e., (1) ÷ (2))
	(1)	(2)	(3)
	gm		
99	242.3	1,150	0.211
100	289.3	1,308	0.221
101	299.1	1,452	0.206
102	276.3	1,417	0.195

[1] For Q/C the standard error of the difference between the dietary means is ± 0.016 (18 degrees of freedom), so that there is no significant difference between the means.

ences between the appetites of the rats receiving the various rations.

The mean Q/C for the 4 rations is 0.208 gm/day/$W^{0.88}$. Taking the metabolizable energy of the rations as 3.65 Calories per gram, a figure calculated from the data of Fraps ('43), then Q would have a mean value of 0.76 Cal./day/$W^{0.88}$ under the conditions of the experiment — e.g., the rats were of the hooded Lister strain, and were caged without bedding in a room kept within the temperature range of 21 to 24°C.

SUMMARY

1. A method is put forward for calculating the food intake of ad libitum-fed rats, in terms of their body weight, as a

measure for the detection of differences in their appetite, according to the treatment they are receiving.

2. In a calculated example, the "appetite quotient" of rats receiving a niacin-deficient diet is equal to that found with niacin-supplemented rations.

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UNSATURATED FATTY ACIDS AND ALLOXAN DIABETES [1]

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ONE FIGURE

(Received for publication July 20, 1953)

It has been shown that dietary factors can influence the toxic and diabetogenic action of alloxan. György and Rose ('48) observed high toxicity of alloxan when the experimental animals were fed diets free from yeast and high in lard or coconut oil, and obtained some reduction in the early mortality by supplementing the diets with tocopherol or yeast. Compared with rats fed a high carbohydrate diet, previous subsistence on a high lard ration has been found to increase the sensitivity of the animals to the toxic and diabetogenic action of alloxan (Martínez, '45; Houssay and Martínez, '47; Rodríguez and Krehl, '52), and also to increase the incidence of diabetes in partially pancreatectomized rats (Martínez, '46; Houssay and Martínez, '48). On the other hand, a high coconut oil diet has been observed to decrease the incidence of both alloxan diabetes (Houssay and Martínez, '47; Rodríguez and Krehl, '52) and pancreatic diabetes (Houssay and Martínez, '48). Evidence is available that the protective action of coconut oil is related to the fatty acid fraction of the oil and not to the unsaponifiable fraction (Martínez, Cattáneo and Houssay, unpublished experiments).

[1] This investigation was supported in part by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. A.

Using diets in which a single fatty acid replaced the natural fat or oil, Rodríguez and Krehl ('52) observed a lowered incidence of alloxan diabetes in rats fed high caprylic or high palmitic acid diets, as compared with those animals fed a high carbohydrate ration. The administration of vitamin E to the animals fed these fat diets was without influence.

The present paper reports data showing that the aggravating action of certain fats is related to their content of unsaturated fatty acids.

TABLE 1

Composition of high carbohydrate and high fat diets used

INGREDIENTS	HIGH CARBOHYDRATE	HIGH FAT
	gm	*gm*
Wheat flour	60	10
Corn flour	51.5	25
Casein	20	20
Yeast	5	5
Salts (McCollum-Davis)[1]	1	1
Cod liver oil	5	5
Fat	..	34
Polyoxyethylene stearate	..	0.5
Water	50	50

[1] J. Biol. Chem., *53:* 55, 1918.

MATERIALS AND METHODS

White male rats from the Institute strain were used. They were distributed among several groups and each group first fed ad libitum for one month one of the experimental diets with the composition given in table 1. It will be noticed that one diet was high in carbohydrate, whereas the other was high in fat. When an oil or a single fatty acid was used, it replaced the 34 gm of fat indicated in the formula for the high fat ration. In these cases an emulsifying agent, polyoxyethylene stearate, was used in the amount shown in table 1.[2] Table 2 shows the calculated fatty acid composition of

[2] Kindly supplied by Dr. W. A. Krehl, Yale Nutrition Laboratory, New Haven, Connecticut.

the different fat diets used. To the groups receiving vitamin E, α-tocopherol acetate was administered, diluted with corn oil and given orally by dropper in an amount equivalent to 5 mg of vitamin E per rat per day. After one month on this regime, the rats were injected with alloxan, intraperitoneally or intravenously, in the doses indicated in table 3.

TABLE 2

Fatty acid composition (calculated) of the fats used

		CAPRYLIC + CAPRIC ACIDS	PALMITIC + STEARIC ACIDS	COCONUT OIL	LARD	OLIVE OIL	OLEIC ACID	CORN OIL	SUNFLOWER OIL	LINSEED OIL
IODINE NUMBER		0.6	1.0	9.0	71.0	84.4	88.3	109.1	130.9	173.8
Saturated	Total					12.1		14.1	8.8	10.0
	Caprylic	37.9		7.9						
	Capric	43.6		7.2						
	Lauric	18.2		48.0						
	Myristic			17.5	1.2					
	Palmitic		46.3	9.0	29.2		1.9			
	Stearic		49.7	2.1	14.1					
Unsaturated	Total	0.3	4.0							
	Oleic			5.7	44.2	77.2	98.1	44.6	30.0	31.9
	Linoleic			2.6	6.5	10.7		41.3	61.2	5.4
	Linolenic									52.7

During the following 10 days, mortality was observed, and immediately after this period the animals were fasted for 7 hours, after which the blood glucose was measured by the Somogy-Nelson method. An animal was considered to be diabetic when under these conditions the blood sugar was 150 mg% or more. All of the rats that died during the first 10 days had hyperglycemia.

All of the animals were given food and water ad libitum and were weighed once a week. The growth of the rats is shown in figure 1.

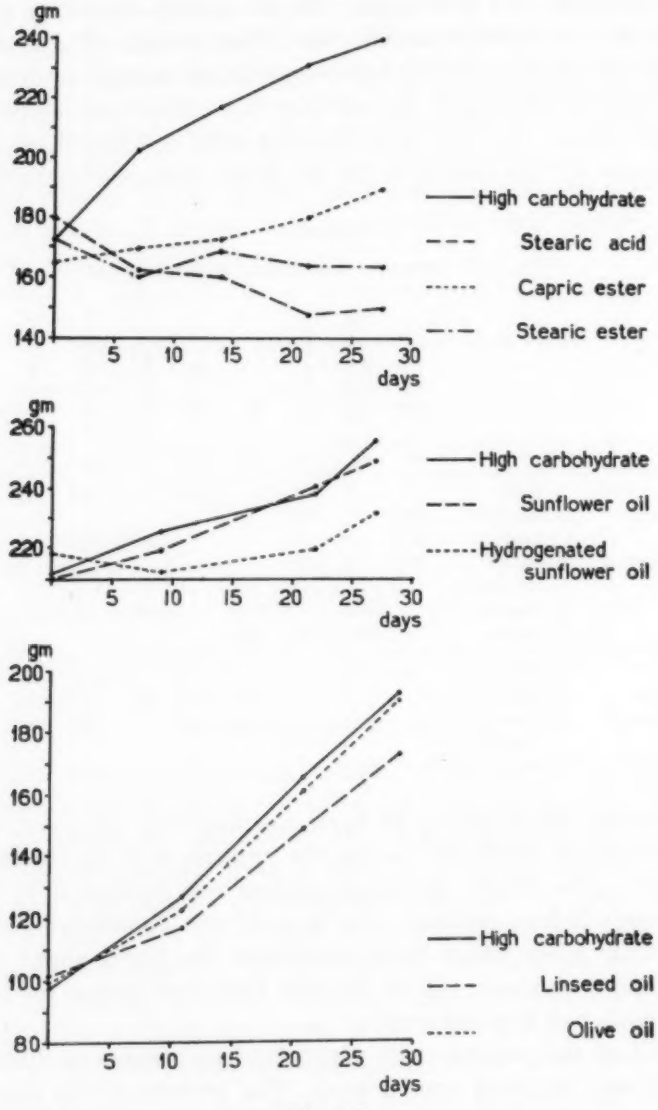
gm
240
220
200
180
160
140
5
10
15
20
25
30
days
High carbohydrate
Stearic acid
Capric ester
Stearic ester
gm
260
240
220
5
10
15
20
25
30
days
High carbohydrate
Sunflower oil
Hydrogenated sunflower oil
gm
200
180
160
140
120
100
80
5
10
15
20
25
30
days
High carbohydrate
Linseed oil
Olive oil

Figure 1

RESULTS

Examination of the data in parts A and B of table 3 shows that diets rich in saturated fatty acids had a markedly protective action against the toxic and diabetogenic action of alloxan. On the other hand, high fat diets rich in unsaturated fatty acids had an aggravating effect.

Diets rich in different oils also influenced the action of alloxan. The incidence of diabetes increased as the proportion of unsaturated fatty acids was raised (see part C of table 3).

In all of the experiments there was a close parallelism between the diabetogenic action of alloxan and the iodine number (degree of unsaturation) of the fats used in the different diets.

From the data in part D of table 3 it is evident that the partial hydrogenation of sunflower oil diminished the toxic and diabetogenic action of alloxan.

The administration of vitamin E to the animals fed a high fat diet rich in oil had no effect on the toxic and diabetogenic action of alloxan. These rats still had a greater incidence of diabetes than those fed a high carbohydrate diet (shown in part E of table 3).

DISCUSSION

The protective action of some fats against the toxic and diabetogenic action of alloxan seems to be related to the quantity of saturated fatty acids present in the fat used. This marked effect was observed not only in those rats fed saturated fatty acids, which are poorly absorbed from the intestine — that is, in the animals fed a high palmitic plus stearic acid diet, on which the rats lost weight during the month previous to the administration of alloxan. The same protective action was also observed in the rats fed diets rich in the ethyl ester of caprylic plus capric acids, a diet on which the animals gained weight. In other words, this protective action occurred whether the animals gained or lost weight.

TABLE 3

Influence of various high-fat diets on toxic and diabetogenic action of alloxan

DIET RICH IN	IODINE NUMBER OF FAT USED	NUMBER OF RATS	MORTALITY	DIABETES	
			Number of rats	No. of rats	%
A. Diets rich in saturated fatty acids (alloxan: 40 mg/kg intravenously)					
Carbohydrate	..	38	5 (13%)	17	44
Caprylic + capric (ethyl esters, 50% each)	0.6	19	0 (0%)	0	0
Palmitic + stearic acids (50% of each)	1.0	39	4 (10%)	5	12
Palmitic + stearic (ethyl esters, 50% of each)	1.0	37	1 (2.7%)	2	5
B. Diets with increasing amounts of unsaturated fatty acids (increasing iodine numbers; alloxan 160 mg/kg intraperitoneally)[1]					
Caprylic acid	0	21	1 (5%)	6	28
Palmitic acid	0	40	9 (23%)	9	22
Coconut oil	9	34	4 (12%)	10	29
Lard	70	28	18 (64%)	21	75
Oleic acid	89	20	11 (55%)	16	80
Corn oil	109	20	18 (90%)	20	100
C. Diets rich in oils and diabetogenic action of alloxan (35 mg/kg intravenously)					
Carbohydrate	..	20		3	15
Olive oil	84	20		4	21
Corn oil	109	19		4	20
Sunflower oil	131	19		5	26
Linseed oil	174	20		7	35
D. Influence of partial hydrogenation of sunflower oil (alloxan 45 mg/kg intravenously)					
Carbohydrate	..	18	4 (22%)	17	94
Sunflower oil	131	20	14 (70%)	20	100
Hydrogenated sunflower oil (melting point 35°C.)	90	18	8 (44%)	12	66
E. Failure of vitamin E administration (5 mg/rat/day) to influence the action of alloxan (40 mg/kg intravenously)					
Carbohydrate		20	3 (15%)	15	75
Olive oil + vitamin E		18	4 (22%)	18	100
Sunflower oil + vitamin E		20	8 (40%)	20	100
Corn oil + vitamin E		20	8 (40%)	20	100

[1] These experiments were performed in the Yale Nutrition Laboratory, Yale University School of Medicine, New Haven, Conn., U.S.A., by R. R. Rodríguez and W. A. Krehl.

In those cases in which the fat of the diet was unsaturated fatty acid — for example, those of the rats fed a high oleic acid diet who gained weight as did those fed a high carbohydrate diet — a marked toxic and diabetogenic action of alloxan was observed.

Animals fed diets containing the oils showed a growth curve similar to those of animals fed a high carbohydrate diet. In this case the diabetogenic action of alloxan was different, and correlated with the iodine number of the oil used. The administration of vitamin E to the animals had no influence on this difference, results which confirm those published earlier by Rodríguez and Krehl ('52).

Another bit of evidence that the toxic and diabetogenic action of alloxan is markedly increased by the unsaturated fatty acids of the diet is the parallelism shown in table 3 between the number of diabetic rats and the iodine number of the fat used. Partial hydrogenation of one of the oils (to a certain degree but not completely, in order not to interfere with its intestinal absorption) was associated with reduced effects of alloxan (part D of table 3).

Studies of the possible mechanism of this action and the effects of unsaturated fatty acids upon diabetes produced by subtotal pancreatectomy in rats are in progress.

SUMMARY

The influence of different diets fed during a preliminary period of one month on the toxic and diabetogenic action of alloxan was studied in male rats. Following this month-long regime, the animals were injected with alloxan and mortality was observed over a 10-day period. Immediately thereafter a 7-hour fasting glycemia was produced and measured.

High fat diets, the fat in which consisted primarily of saturated fatty acids, had a markedly protective action against the toxic and diabetogenic effects of alloxan as compared with a high carbohydrate diet.

High fat diets rich in unsaturated fatty acids had an aggravating action.

Partial hydrogenation of sunflower oil reduced the toxic and diabetogenic action of alloxan.

The administration of vitamin E to the rats fed the high oil diets had no influence on the action of alloxan.

There was a marked and direct correlation between the iodine number of the fat fed and the toxic and diabetogenic action of alloxan.

ACKNOWLEDGMENTS

The authors wish to express their thanks to the following persons and firms who kindly supplied some of the materials used in these experiments: Laboratorios Armour, Mr. J. Estevez (Delbene Hnos y Savia), Molinos Rio de la Plata S. A., Pedro S. Bresciano Ltda., Dr. G. Loew (Gobecia S. A.), Bodegas y Viñedos Giol S. A., Refinerias de maíz and Primera Compañia Argentina de Fermentos.

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NITROGEN BALANCE OF ADULT RATS FED AMINO ACID DIETS LOW IN L-, DL- AND D-TRYPTOPHAN [1]

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ONE FIGURE

(Received for publication July 2, 1953)

The absence of tryptophan in the diet is known to cause negative N balance in adult human subjects (Rose, '49) as well as in adult albino rats (Benditt et al., '50). The optical isomers of tryptophan are reported to be equally effective in promoting the growth of the young rat (Berg and Potgieter, '31-'32; Berg, '34; du Vigneaud, Sealock and Van Etten, '32). Rose ('49) reported, however, that D-tryptophan cannot be utilized by the adult human male for the maintenance of N equilibrium. The experiments described below represent an attempt to discover the minimum quantity of tryptophan required to maintain N balance, under rigidly controlled conditions, in the adult rat.

METHODS

Six groups of adult male albino rats of the Wistar strain were used. Each group initially included 14 to 16 rats, but owing to some deaths, illnesses and errors in feeding or the collection of excreta, the number of animals in any single experiment varied from 6 to 15.

At the beginning, and after a 48-hour fast, the mean body weights for the 6 groups ranged from 195 to 280 gm. The

[1] This work has been supported by the Office of Naval Research.

maximum difference between the lightest and heaviest animals in any group was 50 gm. According to custom in this laboratory, the energy allowance was 121 Cal./day/$kg^{3/4}$. For the type of diets employed and the conditions of these experiments, this caloric intake maintains a relatively constant body weight. Each single experiment included the following sequence of feedings: maintenance diet (9.6% whole egg protein), two weeks; nitrogen-free (N-free) diet, one week; amino acid diet containing approximately half of the maintenance requirement of total nitrogen (half-N), one week; amino acid diet containing twice this amount of total nitrogen (full-N), one week. This 5-week cycle of feeding was repeated for each amino acid mixture investigated.

The basic amino acid mixture simulates the proteins of whole egg in that it contains approximately the same amount of each natural isomer of the essential amino acids per gram of total nitrogen. The non-essential amino acids of egg protein are replaced, in this mixture, by the unnatural isomers of 6 essential amino acids plus sufficient L-glutamic acid to make up the same amount of total nitrogen.[2] This "complete" amino acid mixture and the basal diet in which it is used are described elswhere (Anderson and Nasset, '48; Nasset, Anderson and Siliciano, '51). In the midst of these experiments vitamin B_{12} was added to the diets in sufficient amount to supply each rat approximately 0.1 μg daily. This addition to the vitamin supply had no detectable effect on N balance. The N-free diet was compounded simply by omitting the amino acid mixture and substituting an equal weight of sucrose.

It is the custom in this laboratory to observe changes in nitrogen balance which are brought about by a stepwise reduction of the concentration of one essential amino acid at a time. This process simulates the consecutive testing of a series of proteins of different biological values. It is prob-

[2] All amino acids were purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio.

ably a more physiological procedure than omitting completely one of the essential amino acids from the mixture.

At some point in this stepwise reduction a concentration is found which becomes limiting in the efficient utilization of the total dietary nitrogen. In the present series of experiments, the minimum diminution of tryptophan was to one-third, and the maximum diminution to one-twelfth, of the concentration of tryptophan present in the "complete" amino acid mixture. Several fractions intermediate between one-third and one-twelfth L-, DL- and D-tryptophan were also investigated.

RESULTS

Table 1 contains the average results of all experiments with the rats of series 200. Experiments I and IV of table 1 represent the initial and final 5-week feeding cycles in the whole program of 20 weeks' duration. The diet in experiments I and IV contained the complete amino acid mixture and the results obtained on feeding it conform very closely to many others previously obtained in this laboratory under identical conditions. The noteworthy features of the results with the complete amino acid mixture are: (1) The absence of a rise in urinary nitrogen when nitrogen intake is doubled (compare half-N with full-N data), and (2) the attainment, essentially, of N equilibrium at very low intake of total N. These results indicate that the increment of the complete amino acid mixture, represented by the difference in ingested N between the half-N and the full-N periods, is completely utilized in the improvement of N balance. A numerical expression of this fact is given by K′ in table 1, in which unity indicates complete utilization. K′ is the slope of the line joining the half-N and full-N points when N balance is plotted against N intake. The last item in table 1, NI_e, is the total N intake, in the form of a specific amino acid mixture, computed to be required for the attainment of N equilibrium. This item is the point of intersection of the line represented by K′ with the line representing N equilibrium. It is computed for each animal separately from the N balance data

TABLE 1

Average data for rats receiving amino acid mixture diets

Experiment number	SERIES 200 RATS			
	I	II	III	IV
Amino acid mixture	Complete	1/3 DL-Tryptophan	1/6 DL-Tryptophan	Complete
Number of rats	10	10	10	11
Body weight (kg)	0.280	0.294	0.292	0.296
Metabolic body size ($kg^{3/4}$)	0.385	0.399	0.397	0.401
	N balance data (mg N/day/$kg^{3/4}$)			
Half-N period:				
N intake	73	69	66	69
Fecal N	38	32	30	27
Urinary N	121	110	135	108
N balance	−86	−73	−99	−65
Full-N period:				
N intake	143	144	140	138
Fecal N	35	33	32	28
Urinary N	116	117	145	110
N balance	−7	−5	−37	0
K′ (Half-N; full-N)[1]	1.11 ± 0.08	0.90 ± 0.04	0.85 ± 0.03	0.95 ± 0.06
NI_e[2]	151 ± 4	151 ± 6	184 ± 5	142 ± 7

[1] K′ is the slope of the line joining the half-N and full-N points when nitrogen balance is plotted against nitrogen intake.
[2] NI_e is the nitrogen intake computed to be necessary for attainment of nitrogen equilibrium.

of the half-N and the full-N periods, and this accounts for the fact that the mean values of NI_e do not agree precisely with the values obtained by subtracting the mean N balance from the mean N intake. The reduction in NI_e between control experiments I and IV is quite characteristic when the complete amino acid mixture is fed, and it has been observed, in many other experiments as well, to progress uniformly over a period of at least 6 months. The experiments represented in table 1 extended over a period of 20 weeks.

A reduction in DL-tryptophan to one-third of its concentration in the complete amino acid mixture (experiment II, table 1) failed to cause a decisive diminution in the nutritive value of the amino acid mixture. The only significant difference ($P = 0.03$) between the data for experiment II and those of its adjacent control, experiment I, was in the value of K′. If K′ for experiment II is compared with the mean K′ (1.03) of both control periods, experiments I and IV, the difference is not significant ($P = 0.12$). In nitrogen balance and the value for NI_e, experiments I and II are indistinguishable, and it is concluded that one-third DL-tryptophan is not a low enough concentration to affect adversely the utilization of the total dietary N.

A reduction of tryptophan to one-sixth of the amount in the complete amino acid mixture produces an adverse effect on N balance. As is indicated in table 1, experiment III, the N balance was depressed by approximately equal amounts in half-N and full-N periods. This accounts for the fact that K′ (0.85) for this experiment was not significantly lower than K′ (0.95) for experiment IV ($P = 0.18$). The computed NI_e for the one-sixth DL-tryptophan diet was significantly greater than that for the adjacent control diet ($P = 0.001$).

Table 2 contains the average results of all experiments with the rats of series 280. Of the 25 weeks required to complete this set of experiments, only the middle 5 weeks, experiment III, were devoted to control observations on the feeding of the complete amino acid mixture. Experiment I indicates in a decisive fashion that one-sixth D-tryptophan

is inadequate to permit maximum utilization of dietary N. As a consequence, K′ is only one-third as great as the control value and NI_e is more than doubled. Both of these differences are highly significant ($P<0.001$). In experiment II tryptophan was omitted from the amino acid mixture. The indicated slight average improvement in N balance obtained by doubling the intake of N in the tryptophan-free diet is not significant. Mathematically it is possible to compute both K′ and NI_e and they are entered in table 2, but they probably have little or no physiological meaning except that N balance is severely depressed in the absence of tryptophan regardless of the N intake. Experiment IV shows that one-fifth DL-tryptophan is a concentration which limits normal utilization of dietary N. This fact is demonstrated by the values for K′ and NI_e, both of which are different from corresponding values ($P<0.001$ and <0.04, respectively) obtained in the control period (experiment III, table 2). As judged by K′, NI_e and N balance, the one-fifth DL-tryptophan mixture is not so severely limiting as one-sixth DL-tryptophan (experiment III, table 1).

Early in this work it became evident that the feeding of amino acid mixtures low in tryptophan might yield values for K′ which are not different from normal. This fact is illustrated in table 1. Experiment III shows that one-sixth DL-tryptophan depressed the N balance, but since the depression was approximately equal for both half-N and full-N periods, the value of K′ was not different from that in either experiment II or experiment IV ($P=0.32$ and 0.18, respectively).

The computed total N required for the attainment of N equilibrium, NI_e, was significantly increased, however, in both experiment III, table 1, and experiment IV, table 2 ($P<0.001$ and <0.04, respectively). This relationship is shown for all experiments with all series of rats in figure 1, in which NI_e is plotted against actual tryptophan intake. Each point represents the average of results obtained from 6 to 15 animals. One of the most striking features of this figure is the abrupt

TABLE 2

Average data for rats receiving amino acid mixture diets

Experiment number	SERIES 280 RATS				
	I	II	III	IV	V
Amino acid mixture	1/6 D-Tryptophan	No tryptophan	Complete	1/5 DL-Tryptophan	1/9 L-Tryptophan
Number of rats	15	9	11	10	8
Body weight (kg)	0.245	0.258	0.262	0.274	0.276
Metabolic body size ($kg^{3/4}$)	0.348	0.362	0.366	0.379	0.381
	N balance data (mg N/day/$kg^{3/4}$)				
Half-N period:					
N intake	75	72	71	65	67
Fecal N	35	34	36	34	36
Urinary N	122	131	122	114	118
N balance	−81	−93	−88	−83	−87
Full-N period:					
N intake	149	138	138	133	130
Fecal N	35	37	35	31	35
Urinary N	167	191	100	117	139
N balance	−52	−90	+3	−15	−44
K'[1]	0.46 ± 0.06	0.21 ± 0.05	1.35 ± 0.06	1.01 ± 0.06	0.75 ± 0.09
NI_e[2]	299 ± 27	1142 ± 416	136 ± 3	150 ± 6	220 ± 39

[1] K' is the slope of the line joining the half-N and full-N points when nitrogen balance is plotted against nitrogen intake.
[2] NI_e is the nitrogen intake computed to be necessary for attainment of nitrogen equilibrium.

rise in NI_e when the intake of tryptophan, in any form, falls below approximately 7 mg/day/$kg^{3/4}$ (approximately 2.5 mg/day/250-gm rat). Another feature, to be discussed later, is the superiority of the natural isomer over either the unnatural isomer or the racemic mixture.

The tryptophan requirements for maintenance of N equilibrium in the adult male rat, under the rigidly controlled conditions of this experiment, are summarized in table 3. Each series number refers to a single group of rats. Owing to illness, deaths and errors in feeding or collection of ex-

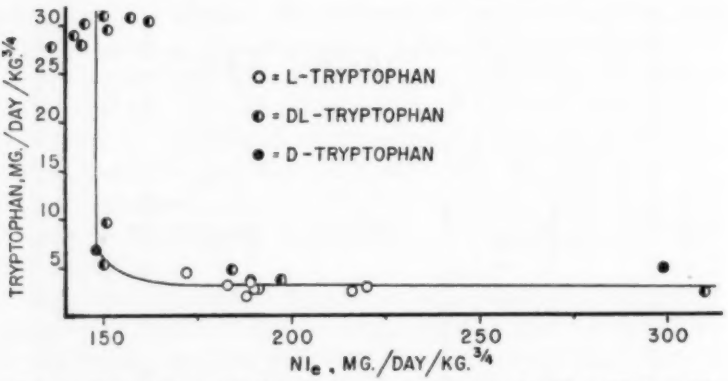

Fig. 1 Plot of computed dietary nitrogen requirement for N equilibrium, NI_e, against tryptophan intake. Each point is a mean obtained with 6 to 15 animals.

creta, the number of rats varied from one experiment to the next. The experiments represented in table 3 include only those in which the tryptophan concentration was limiting and in which half-N and full-N feedings were employed. From the two points thus obtained experimentally the total nitrogen required for N equilibrium, NI_e, was computed. Since the composition of the diet is precisely known and tryptophan was the limiting amino acid, it is possible also to compute the tryptophan requirement for maintenance of N equilibrium. These figures are entered in the last three columns of table 3. It is evident that the L-tryptophan requirement, 4.4 $\pm$ 0.22 mg/

day/kg$^{3/4}$, is significantly less than the DL-tryptophan requirement, 6.0 ± 0.31 mg/day/kg$^{3/4}$ ($P<0.0003$), which in turn is less than the D-tryptophan requirement, 8.8 mg/day/kg$^{3/4}$ ($P<0.0003$). Thirty-five rats were fed the natural isomer, 30 were fed the racemic mixture, and 26 received the unnatural isomer. It is striking that the highest mean value in the L-group was lower than the lowest mean value in the DL-group. The same comparison holds between the DL- aud the D-groups.

TABLE 3

Tryptophan requirement of adult male rats

SERIES	AMINO ACID MIXTURE	NUMBER OF RATS	ACTUAL INTAKE ON FULL-N	MG TRYPTOPHAN/DAY/KG$^{3/4}$		
				Computed requirement of isomers for N equilibrium		
				L	DL	D
200	1/6 DL[1]	10	4.9		6.4 ± 0.16[2]	
220	1/12 DL	10	2.4		5.4 ± 0.28	
	1/12 L	6	2.5	3.8 ± 0.11		
280	1/6 D	15	5.0			9.8 ± 0.29
	1/5 DL	10	5.4		6.1 ± 0.24	
	1/9 L	8	3.0	5.1 ± 0.28		
360	1/10 L	8	2.8	3.9 ± 0.09		
	1/8 L	13	3.3	4.6 ± 0.10		
	1/4 D	11	6.8			7.5 ± 0.21
Mean				4.4 ± 0.22	6.0 ± 0.31	8.8 ± 0.57
Percentage of mean L-tryptophan requirement				100	136	200

[1] Isomer and approximate amount of tryptophan as compared with "complete" amino acid mixture.

[2] Standard error of the mean.

DISCUSSION

The results presented above permit, for the first time, a quantitative estimate of the L-, DL- and D-tryptophan requirements of the adult rat for maintenance of N equilibrium. Judging from the results in table 3, there can be no doubt that the natural isomer is used to greatest advantage. On the basis of these experiments, the animals require twice as much D- as L-tryptophan to achieve N equilibrium. Theoretically

the DL-mixture should fall in the middle, between the optical isomers, with a computed requirement which is 150% of the L-requirement. The actual mean value, 6.0 ± 0.31 mg/day/$kg^{3/4}$, was 136%, which is not far enough from theory to upset this idea. On the other hand, the low figure may represent a true favorable effect of the natural isomer on the metabolism of the unnatural one. It should be noted, however, that the series 220 rats (table 3) were fed the same concentrations of L- and DL-tryptophan, and the computed requirement of the racemic mixture in that case was 142% of the requirement of the natural isomer.

A possibility exists that the superiority of L-tryptophan may be due in part to the fact that the natural isomers of amino acids appear to be preferentially absorbed from the intestine. Gibson and Wiseman ('51) observed the disappearance of both isomers from the intestines of rats after introducing the racemic mixture. In trials with 13 amino acids, the L-isomer invariably disappeared faster than the D-isomer. Unfortunately, tryptophan was not included in these experiments. Quite apart from any possible disadvantage in absorption, it seems only logical that the D-isomer of any amino acid, if utilized at all, should be less efficiently utilized than the corresponding L-isomer. The hazards of deamination, formation of the keto acid, and reamination could scarcely be encountered without some loss. Other evidence in support of this view was presented in a previous publication (Nasset and Siliciano, '52).

Under the conditions employed in the present experiments, it is evident that D-tryptophan can be utilized for the maintenance of nitrogen balance but with approximately one-half the efficiency of L-tryptophan. It is unjustifiable to conclude, however, that they are nutritionally even approximately equivalent. The animals ingesting the unnatural isomer were decidedly more unkempt and listless than the same or other animals ingesting the natural isomer. These signs may indicate a disadvantage due to ingestion of D-tryptophan which is not manifested in a disturbance of N balance.

In a discussion of dietary limitation of tryptophan the supply of niacin must be considered. As a matter of course niacin has been included in all of the diets fed in this investigation in sufficient amount to furnish the average adult rat (250 gm) with approximately 0.4 mg of this vitamin per day. As an additional precaution, however, the niacin was doubled in three low-tryptophan experiments carried out with the rats of series 360. It is evident from the data of table 3 that the additional quantity of niacin had no effect on the results. Vitamin B_{12} was added (approximately 0.1 μg/rat/day) for the first time in series 360. Experiments I and V in this series were made with the complete amino acid mixture and the usual amount of niacin and the results were quite typical in every respect. It is concluded, therefore, that a deficiency of either one of these vitamins in any of these experiments is unlikely.

Accepting certain assumptions, it is possible and interesting to compare the tryptophan requirement of the adult rat with that of the adult human being. Rose ('49) states that two of his human subjects were maintained consistently in positive N balance on an intake of 0.15 gm of L-tryptophan per day. On the assumption that his subjects weighed 70 kg, the L-tryptophan requirement is 6.2 mg/day/$kg^{3/4}$. Since this amount was enough to maintain positive N balance, it seems likely that the actual requirement for N equilibrium is less and it must, therefore, agree quite well with the mean requirement of the rat, 4.4 mg/day/$kg^{3/4}$, as indicated in table 3. Among his 31 subjects, however, Rose found one who required 0.25 gm of L-tryptophan per day and he prefers to regard this as the minimum requirement, which is 10.3 mg/day/$kg^{3/4}$. If the same reasoning is applied to the present experiments, it is discovered that one rat, of the 35 fed L-tryptophan, required 10.8 mg/day/$kg^{3/4}$. This may be pure coincidence, but it seems more likely that it indicates that, on a comparable basis, the L-tryptophan requirement for the adult male of these two species is the same. Another and an inevitable consequence of Rose's reasoning is that the greater

the number of subjects investigated, the greater the likelihood that the "minimum" will increase.

SUMMARY

Nitrogen balance was determined on a group of adult male albino rats which derived all of their dietary nitrogen from mixtures of amino acids. Each experiment included the following dietary regimens in the order given: 14 days on maintenance diet (9.6% whole egg protein); 7 days on N-free diet; 7 days on amino acid diet supplying approximately half of the maintenance requirement of total nitrogen; 7 days on double the quantity of the amino acid mixture fed in the previous period. These diets, except the maintenance diet, were fed by stomach tube in two equal portions daily, and each rat received the same amount of diet each day.

If the quantity of tryptophan in the amino acid mixture is reduced sufficiently, the N balance is adversely affected. When tryptophan is the limiting factor in the utilization of total dietary N, it is assumed that N balance is a linear function of tryptophan intake. On this assumption, the requirement for maintenance of N equilibrium is 4.4 ± 0.22 mg of L-, 6.0 ± 0.31 mg of DL-, and 8.8 ± 0.57 mg of D-tryptophan/day/$kg^{3/4}$.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Vernon Gatewood and Miss Marion White for their technical assistance.

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THE EFFECT OF ANTIBIOTICS ON THE GROWTH OF CHICKS RAISED IN NEW QUARTERS[1]

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(Received for publication August 6, 1953)

It has been known since 1946 (Moore et al.) that antibiotics stimulate the growth of chicks. However, Coates and associates ('52) showed that antibiotics, at the levels fed, were less effective in new quarters than in those areas in which chicks had been reared constantly. These results were confirmed by Bird et al. ('52) and Hill et al. ('52).

The present investigations were undertaken in order to study further the effect of antibiotics on the growth of chicks raised in new quarters.

EXPERIMENTAL

New batteries with raised wire floors were used in these studies. The batteries were located in a temperature-controlled room which had not been used previously for housing chicks.

New Hampshire chicks of mixed sex obtained from a commercial hatchery were weighed and placed on experiment at one day of age. Feed and water were supplied ad libitum. Ninety chicks were used per lot in the first experiment, 33 in experiments 2 and 3.

Crystalline aureomycin hydrochloride was used as the antibiotic.

RESULTS AND DISCUSSION

The results of three experiments are summarized in table 2. In agreement with the previously cited work, it was found

[1] Contribution from the Department of Poultry Science, North Carolina Agricultural Experiment Station, as Journal Paper 500.

in experiment 1 that the addition of 10 mg of aureomycin per pound of ration did not increase the growth of chicks raised in the new quarters, although this level is more than adequate to produce a growth response in chicks raised in old quarters (Kramke and Fritz, '51).

The second experiment was undertaken to ascertain whether or not a growth response to antibiotics could be obtained if the pens were not cleaned thoroughly between experiments.

TABLE 1

Composition of basal ration

INGREDIENTS	AMOUNT
	%
Soybean meal (44% protein)	30
Yellow corn	56
Fish meal	3
Whey product	3
Alfalfa meal	3
Salt	0.3
Defluorinated phosphate	2.0
Limestone	1.5
Methionine	0.2
Vitamin and mineral mix [1]	1.0

[1] Supplied, per pound of ration, thiamine HCl 0.9 mg, riboflavin 1.6 mg, calcium pantothenate 5.3 mg, niacin 8.0 mg, pyridoxine HCl 1.6 mg, biotin 45 gm, folic acid 0.45 mg, B_{12} 1 gm, choline chloride 700 mg, inositol 450 mg, alpha-tocopherol acetate 10 mg, menadione 10 mg, vitamin A, 3,000 units; vitamin D, 180 units, $CoCl_2 \cdot 6H_2O$, 0.9 mg; $ZnCl_2$ 2.25 mg; $CuSO_4$, 4.5 mg; $MnSO_4$, 45 mg; $FeSO_4 \cdot 7H_2O$, 90 mg; KI 0.66 mg; $MgSO_4$, 2.25 gm, KCl 2.25 gm.

TABLE 2

The effect of normal and high levels of antibotics on the growth of chicks in new quarters

ANTIBIOTIC	EXPERIMENT NO.		
	1	2	3
		4-wk. wt., gm	
None	374	360	376
Aureomycin, 10 mg/lb.	388	369	375
Aureomycin, 100 mg/lb.		401 [1]	407 [2]

[1] Significant past 1% level, "t" = 4.170.
[2] Significant past 5% level, "t" = 2.271.

The dropping pans of the battery which had been used in experiment 1 were washed out with hot water but the pens were not otherwise cleaned. The battery was placed in the room in which experiment 1 had been carried out. Since Hill et al. ('52) found that high levels of antibiotics gave some growth response in new quarters, a very high level (100 mg/lb.) of aureomycin was also included in the test.

Again, as in the first experiment, there was no growth response to 10 mg of aureomycin per pound of ration. However, a highly significant weight increase resulted when the level of aureomycin was increased to 100 mg per pound of ration.

In order to determine whether or not thorough cleaning of the pens and a new environment would prevent the high level of antibiotic from producing a chick growth response, experiment 2 was repeated. In this third experiment, the pens of a battery which had been used previously were thoroughly cleaned with hot water and then washed with steam. The battery was placed in another temperature-controlled room which had not previously housed chicks. New cans were used to hold the feed.

Under these clean conditions there was no response to the lower level of antibiotic, while again 100 mg of aureomycin per pound of ration significantly increased the weight of the chicks.

The results of the three experiments clearly demonstrate that a level of aureomycin sufficient to produce a growth response in old quarters is ineffective in new quarters. A much higher level, however, produced a significant increase in chick growth at 4 weeks of age in both clean and unclean pens. The increased growth brought about by this high level of antibiotic was apparent, although not significant statistically, at one week of age in both experiments 2 and 3.

The explanation of the results obtained in these studies cannot be stated with any degree of certainty. It is possible that the organisms responsible for the decreased growth of chicks raised in new quarters are more antibiotic-resistant

than the growth-retarding organisms present in old quarters. Thus, a higher concentration of antibiotic may be required to overcome the effect of the organisms in new quarters. However, until the mode of action of antibiotics in stimulating chick growth is more fully understood, all such explanations are speculative.

SUMMARY

Aureomycin at a level of 10 mg per pound of ration did not increase the 4-week weights of chicks raised in new quarters. When the level of the antibiotic was increased to 100 mg per pound of ration, however, a significant increase in growth occurred, in both clean and unclean pens.

ACKNOWLEDGMENTS

The aureomycin used in these studies was supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories, Pearl River, New York.

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